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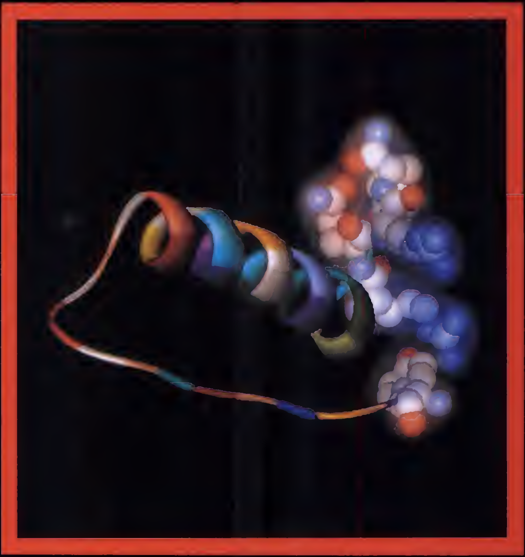

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This is to acknowledge that the slide shown on the front cover of this journal was kindly supplied by Dr. Yi Li, Bristol-Myers Squibb Computer-Assisted Drug Design. The cover slide shows a ribbon rendered model of Neuropeptide Y and shows the space-filled residues of Tyr1 and Arg33-Tyr36. These residues have shown to be those responsible for recognition by the NPY1 receptor.

This One
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Aims and Scope

Current Pharmaceutical Design publishes timely in-depth reviews covering all aspects of current research in rational drug design. Each bi-monthly issue is devoted to a single major therapeutic area. A Guest Editor who is an acknowledged authority in a therapeutic field has solicited for each issue comprehensive and timely reviews from leading researchers in the pharmaceutical industry and academia. Each thematic issue of ***Current Pharmaceutical Design*** covers all subject areas of major importance to modern drug design, including: medicinal chemistry, pharmacology, drug targets and disease mechanisms.

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PREFACE

Welcome to the 1998 Neuroscience edition of *"Current Pharmaceutical Design"*. In this issue I have included a number of chapters which represent therapeutic opportunity rather than more mature areas from which numerous clinical candidates have been selected. Of course, in selecting this type of review, the focus is necessarily less on the medicinal chemistry since few compounds exist or have been published and more on the molecular biology and pharmaceutical opportunity.

Of the five chapters which comprise this issue and meet the general objective of defining new opportunity, the chapter from Karin Briner and Richard Dodel, outlining new approaches to the development of rapid onset antidepressants, details a wide array of compounds which have potential in the treatment of this serious and prevalent disease. It is interesting to observe that even in an area as mature as depression, new and significant opportunity exists.

The remaining chapters deal with a wide diversity of subjects including: oleamide, a new sleep- inducing lipid amide and its catabolising enzyme FAAH which is reviewed by Dale Boger; the inhibitory glycine receptor and integral ion channel, which has potential for the treatment of neuromuscular disorders such as spasticity as discussed by Cord-Michael Becker and Hans-Georg Breitinger; neuroinflammation and its likely central role in critical areas of such medical need as Alzheimer's disease written by Sheryl Hays; and lastly, obesity, which afflicts the Western world's population to an alarming extent and is the major risk factor for late onset diabetes. This last chapter was written by Graham Poindexter, Ildiko Antal Zimanyi and Zahra Fathi.

I hope that you find these articles to be stimulating and thought provoking.

Graham Johnson

New Approaches to Rapid Onset Antidepressants

Karin Briner[1]* and Richard C. Dodel[2]

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Abstract: The challenges for the design of new antidepressant drugs are threefold: rapid onset of antidepressant response, broader efficacy, and fewer adverse effects. While progress has been made to reduce side-effects, the currently available drugs show no convincing evidence for a shorter delay of onset nor for improved efficacy. This review summarizes some of the hypotheses that try to explain the delayed onset and that are currently used for antidepressant drug design. These approaches include combinations of SSRI activity with either NA reuptake inhibition or 5-HT$_{1A}$-, 5-HT$_2$-, or α_2-adrenergic receptor specificity, as well as targeting interactions with specific receptor subtypes (5-HT$_{1A}$, 5-HT$_{1A}$/5-HT$_2$, 5-HT$_{1B/D}$, α_2/5-HT$_2$, D2/D3). Structure activity relationship studies to obtain molecular entities with the desired pharmacological profile are discussed. Many structural modifications have exploited successfully the acquired knowledge on structural features that are necessary for monoamine reuptake inhibition as well as for affinities at various receptors. Most of the current drug design efforts are focused on these approaches, which are based on the monoamine theory of depression; however, novel biological concepts of depression involving other receptor systems or intracellular targets are briefly mentioned. Although their impact on the onset of antidepressant response is only a matter of speculation at this moment, they may lead to the identification of targets for truly novel antidepressants.

The discovery of the tricyclic antidepressant (TCA) imipramine and the monoamine oxidase inhibitor (MAOI) iproniazid in the 1950s initiated the rational approach to the therapy of depression [1, 2]. Subsequently, an abundant number of compounds similar to imipramine and iproniazid were developed by relatively conservative modifications of their molecular structures [3, 4]. This first generation of antidepressants is associated with serious side- effects and toxicity in overdose. Therefore, new antidepressants ("second generation antidepressants") were developed with the focus on selectivity in their pharmacology [5]. These newer agents, such as selective serotonin reuptake inhibitors (SSRIs), reversible monoamine oxidase inhibitors (RIMAs), and noradrenergic reuptake inhibitors are devoid of anticholinergic and cardiovascular side-effects characteristic of TCAs. They do, however, not represent an improvement over older antidepressants in terms of broader efficacy and reduction of the latency before the onset of clinical effects. Indeed, SSRIs are perceived to be less effective than TCAs in more severe forms of depression [6]. They have also brought their particular pattern of adverse reactions due to non-selective stimulation of the postsynaptic 5-HT receptors [7]. More recently, drugs with a rationally designed polypharmacological profile are being pursued. These approaches have been summarized in several excellent reviews [5-15].

The goals of a new generation of antidepressants is to improve treatment in at least three respects [8, 16]: 1. Shorter latency before the onset of clinical effects. 2. Broader efficacy.It is estimated that less than 70% of moderately to severely depressed patients respond to the first antidepressant [17]. 3. Fewer adverse effects. In respect of reduction of adverse effects, the polypharmacological approach targeting specific receptor blocking drugs shows some promise [18]. In this article we will focus on current strategies to achieve a more rapid onset of antidepressant effect and their underlying neurobiological concepts which are essential for a rational drug design.

The Onset of Antidepressant Action

It has become a general belief that antidepressants require several weeks (2-6 weeks) of continuous administration before their mood-elevating properties are fully manifest [19]. It is for this reason that guidelines of the European Community for investigating the efficacy of antidepressants in treating the acute phase of major depression recommend that such studies should have a duration of 4-6 weeks [20]. Such a delayed onset of action is supported by findings from placebo-controlled clinical trials [21]. It has been explained in terms of longer-term adaptive changes in receptor sensitivity and/or density, still, the exact mechanisms responsible for this phenomenon are unclear [22-24].

Nevertheless, there are several lines of clinical evidence that a rapid antidepressant response is possible. Electroconvulsive treatment improves depressive symptoms sooner than do antidepressant drugs [25]. A rapid antidepressant effect is observable following a one night sleep deprivation, which produces an immediate but short-lasting therapeutic effect in about 60% of depressed patients [26]. Normal mood returns within 48 h after cessation of the tryptophan depletion paradigm in drug-remitted patients [27].

Recently, rapid onset of action has been claimed for new types of antidepressants based on studies from preclinical

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Table 1. Treatment of Depression and Early Onset of Action: Proposed Pharmacological Mechanisms and Compounds/Drugs Under Investigation

Mechanism	Examples	Clinical Trials	Clinical Indication for Early Onset
SSRI/5-HT$_{1A}$ antagonist	combination therapy: fluoxetine/pindolol paroxetine/pindolol	[50, 51]	(+)[b)]
SSRI/5-HT$_{1B/D}$ antagonist	n.a.[a)]	n.a.	n.a.
SSRI/5-HT$_{2A}$ antagonist	nefazodone	[92]	-
SSRI/α_2 antagonist	A80426	n.a.	n.a.
SNRI	venlafaxine	[30]	(+)[c)]
5-HT$_{1A}$ agonist/5-HT$_2$ antagonist	flibanserin (BIMT 17)	n.a.	n.a.
5-HT$_{1B/D}$ antagonist	n.a.	n.a.	n.a.
α_2-/5-HT$_2$/5-HT$_3$ antagonist	mirtazapine	[15]	(+)[b)]
D2/D3 agonist/SSRI	roxindole	[125]	(+)[b)]
DA reuptake inhibitor	amineptine	[29, 127]	(+)[b)]

[a)] not available. [b)] None of these studies have been designed for rapid onset determination. [c)] Rapidly escalating dosages have been used in these trials.

experiments and clinical trials [28-31]. These data, though, must be viewed with caution, given the methodological difficulties [32-34] in determining and defining the onset of action of antidepressants [35, 36] and the scarcity of studies specifically addressing this point [33, 37]. Moreover, the use of loading or escalating doses in some studies complicate the interpretation of the results [29, 30]. So far, no compound has been convincingly and unequivocally shown to shorten the lag period [36].

Strategies for the Design of Rapid Onset Antidepressants

The monoamine theory which postulates that depression arises as a result of reduced availability of noradrenaline (NA) and serotonin (5-HT) in the brain, has remained the prevailing basis for the design of new antidepressant drugs. Although acute administration of antidepressants increases the amount of synaptic neurotransmitters soon after administration, an antidepressant effect is observed only after several weeks of treatment. This delayed onset of action may be explained by time-dependent adaptive changes in receptor sensitivity or receptor density. In this context, several physiological concepts have been proposed as approaches to drugs with a potential rapid onset of therapeutic effect (Table 1). The structural features that determine monoamine reuptake or oxidase inhibition as well as affinities at various receptors, are rather well established [5,38-40].

Approaches Involving the 5-HT$_{1A}$ Receptor

The 5-HT$_{1A}$ receptor appears to play a major role in mediating antidepressant response [41]. Postsynaptic 5-HT$_{1A}$ receptors, together with other postsynaptic receptor subtypes, convey neurotransmission at target sites, such as the hippocampus and cortex [42]. Presynaptic 5-HT$_{1A}$ autoreceptors located at somatodendritic sites control neuronal firing [43].

SSRIs/5-HT$_{1A}$ Antagonists

The delayed onset of action may be explained by an activatior of somatodendritic 5-HT$_{1A}$ autoreceptors which reduces cell firing activity which in turn limits the amount of synaptic 5-HT. Upon chronic administration the inhibitory effect of SSRI on the 5-HT neuronal firing is decreased, probably due to desensitization of the 5HT$_{1A}$ autoreceptors. A 5-HT$_{1A}$ antagonist in combination with an SSRI (or MAO inhibitor), is, therefore, expected to hasten the therapeutic effects [44]. This hypothesis has been supported by various animal experiments with more (WAY100635, UH-301) or less ((-)pindolol; (-)-tertatolol) selective 5-HT$_{1A}$ antagonists [45-49]. In addition, in two recent double-blind, placebo controlled clinical studies, in which the 5-HT$_{1A}$ antagonist pindolol was administered in combination with either paroxetine [50] or fluoxetine [51] a greater percentage of patients experienced a therapeutic response and the median time needed to achieve a sustained response (50% reduction of the symptoms) was significantly reduced.[1] Remarkably, the combination of pindolol with the tricyclic drugs desimipramin and trimipramine, that do not block the reuptake of 5-MT, resulted in only one of ten patients achieving a 50% improvement after 28 days compared to eight out of ten patients on the combination fluoxetine/pindolol [55].

These findings have given a strong impetus to the search for selective and full 5-HT$_{1A}$ receptor antagonists [56, 57].

A potential disadvantage of using 5-HT$_{1A}$ antagonists is the possible blockade of postsynaptic 5-HT$_{1A}$- receptors [42]. But animal experiments suggest that it is unlikely that the benefit from enhancing presynaptic 5-HT neurotransmission is canceled by blockade of postsynaptic receptors [58].

---

[1] Although pindolol has a higher affinity for the β-adrenoceptor than for the S-HT$_{1A}$ receptor, there is evidence against an involvement of β-adrenoceptors in the response to pindolol [52-54]. A recent study implies some 5-HT1A agonist character for pindolol [47b].

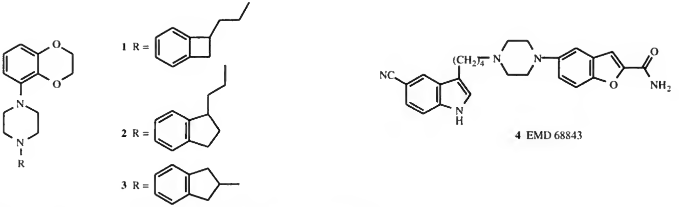


Fig. (1). Presynaptic 5-HT_{1A} receptor agonists and/or postsynaptic 5-HT_{1A} antagonists.

Selective Agonists for Postsynaptic 5-HT_{1A} Receptors (Fig. (1) and (Fig. (2)).

An agonist selective for postsynaptic 5-HT_{1A} receptors could rapidly produce a net increase in neurotransmission, since it would not induce an initial suppression of firing activity of 5HT neurons [59]. Selective postsynaptic $5HT_{1A}$ receptor agonists have not been described so far. Nevertheless, there is indication for a pharmacological difference between presynaptic and postsynaptic 5-HT_{1A} receptors, although the nature of this difference is not well understood yet [60-62]. However, it appears easier to find compounds that are antagonists at the postsynaptic 5-HT_{1A} and agonists at the presynaptic 5-HT_{1A} receptor. For instance, the benzodioxopiperazines 1-3 display postsynaptic 5-HT_{1A} antagonist activity while acting as agonists at the presynaptic 5-HT_{1A} autoreceptors [63]. Similarly, EMD 68843 (**4**) is a 5-HT reuptake inhibitor with selective presynaptic 5-HT_{1A} agonist properties (Fig. (**1**)) [64]. Several general 5-HT_{1A} agonists are in late phases of development [65]. The partial 5-HT_{1A} agonists ipsapirone [66] (**5**), gepirone [67] (**6**), and tandospirone [68] ((±) **7**) are all derivatives of 1-pyrimidylpiperazine (1-PP) and are structurally and functionally related to the marketed anxiolytic buspirone (**8**) [69] (Fig. (**2**)). Their common metabolite 1-PP is an α_2-adrenoceptor antagonist [5, 65].

The benzodioxopiperazine flesinoxan (*R*)-**9** is a 5-HT_{1A} full agonist (K_i (5-HT_{1A}) = 1.7 nM) [70]. It has shown antidepressant activities in preliminary clinical trials; however, no evidence for a rapid onset of action was found [71, 72]. Another 5-HT_{1A} full agonist is alnespirone (*S*)-**10** [73], whose structure is a hybrid based on a series of benzopyran derivatives and buspirone. It has shown antidepressant and anxiolytic activity in animals [74].

The verification of the hypothesis that a selective postsynaptic 5-HT_{1A} agonist (perhaps in combination with a selective presynaptic 5-HT_{1A} antagonist) accelerates onset of therapeutic effect, will depend, at last, on the availability of selective compounds.

One drawback of this approach might be that 5-HT_{1A} agonists only increase neurotransmission at synapses which express this receptor subtype and might be less potent than drugs which have activity for several subtypes.

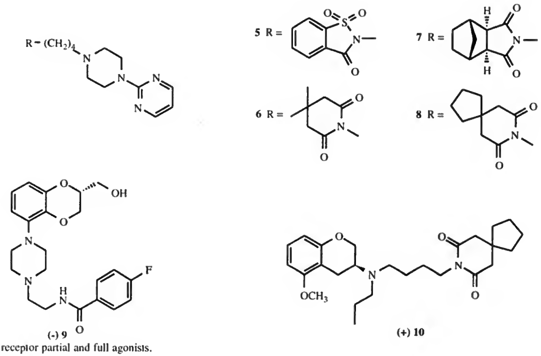
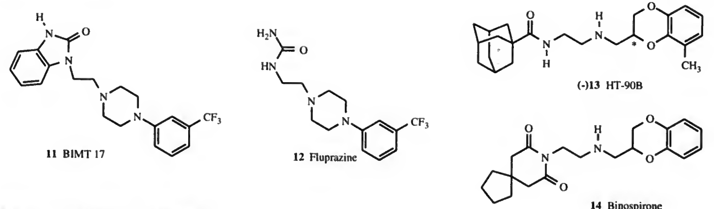


Fig. (2). 5-HT_{1A} receptor partial and full agonists.



11 BIMT 17

12 Fluprazine

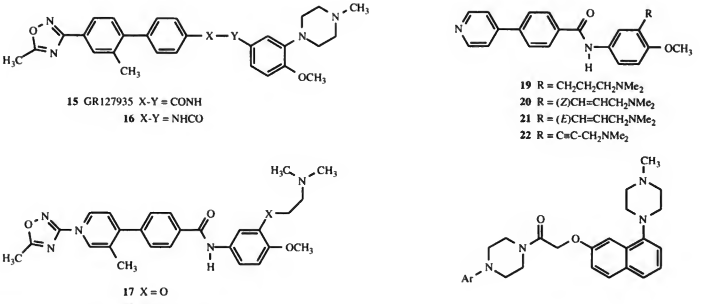
(-)**13** HT-90B

14 Binospirone

Fig. (3). 5-HT_{1A} agonists/5-HT_{2A} antagonists.

5-HT_{1A} Agonists/5-HT_{2A} Antagonists (Fig. (3))

The hypothesis of a balance between $5HT_{1A}$- and 5-HT_2-mediated receptor function as a necessary requirement for rapid onset of action [75] has stimulated the synthesis of several compounds with 5-HT_{1A} agonist and $5HT_2$ antagonist activity.



15 GR127935 X-Y = CONH
16 X-Y = NHCO

19 R = $CH_2CH_2CH_2NMe_2$
20 R = (*Z*)$CH{=}CHCH_2NMe_2$
21 R = (*E*)$CH{=}CHCH_2NMe_2$
22 R = $C{\equiv}C\text{-}CH_2NMe_2$

17 X = O
18 X = CH_2

23

Fig. (4). 5-$HT_{1B/1D}$ receptor antagonists or partial agonists.

The importance of 5-HT_2 receptors with regard to the clinical effects of antidepressants may be related to several studies that documented a reduction of the 5-HT_2 receptor number in several brain areas, including the hippocampus, by long-term antidepressant drug treatment [76].

Flibanserin (BIMT 17; **11**) is a full agonist at 5-HT_{1A} receptors (pK_i = 7.72) and an antagonist at the 5-HT_{2A} receptor (pK_i = 6.90), with no appreciable affinity for other 5-HT receptors, including 5-HT_{2C} (pK_i = 5.40) [77, 78]. It is currently in clinical trials. The structurally related fluprazine (**12**), on the other hand, binds rather poorly to 5-HT_{1A} and $5HT_{1B}$ receptors [5].

HT-90B ((-)**13**) is another selective 5-HT_{1A} agonist/5-HT_{2A} antagonist with high affinity for the 5-HT_{1A} receptor (K_i = 0.18 nM) and the 5-HT_{2A} receptor (K_i = 9.2 nM) [79]. While it displays little affinity for the 5-HT_{2C} receptor (K_i = 260 nM), it has affinity at the α_1 (K_i = 49 nM) and D_2 receptors (K_i = 54 nM). Structurally, HT-90B is related to binospirone (**14**), which is a selective partial agonist at the 5-HT_{1A} receptor [80]. Currently, HT-90B in clinical trials as an anxiolytic and antidepressant.

So far, no convincing data showing rapid onset of antidepressant response are available.

5-$HT_{1B/1D}$ Antagonists[2] *Fig. (4))*

It has been proposed that selective antagonists for the 5-$HT_{1B/1D}$ receptors (probably more precisely 5-HT_{1B} autoreceptors) that control 5-HT release, might act as potentially fast-acting antidepressant drugs, alone or in combination with SSRI-activity [14, 82]. Acute blockade of the terminal 5-HT autoreceptor markedly enhanced 5-HT release in brain slices prepared from human and rodents [83, 84]. Both auto- and hetero-presynaptic 5-HT_{1B} receptors have been shown to be desensitized after long-term treatment with antidepressants. 5-HT_{1B} receptor stimulation has blocked the antidepressant-like effects of SSRIs, such as citalopram and fluoxetine, in two animal studies. Few selective compounds are available as yet to explore this receptor for therapeutic purposes [85]. Another complication is the marked pharmacological differenc between the rat and human 5-HT_{1B} receptors, arising from one amino acid modification [86, 87].

---

[2] A revised nomenclature has been introduced by the Serotonin Club Nomenclature Committee [81], the human 5-$HT_{1D\alpha}$ receptors are now termed 5-HT_{1D} and the 5-$HT_{1D\beta}$ receptors are termed 5-HT_{1B}, with distinction between the human (h5- HT_{1B}) and the rat (r5-HT_{1B}) receptors to account for their pharmacological differences.

The benzanilide GR127935 (**15**) has high *in vitro* affinity for the 5-$HT_{1B/1D}$ receptors (pK_i (r5-HT_{1B}) = 8.5; pK_i (5-HT_{1D}) = 8.5) and 100-fold selectivity over the 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2C} receptors [88]. The SAR study on a series of benzanilides showed that a biaryl moiety was necessary for this high affinity and selectivity [88]. Interestingly, the reversed amide **16** showed reduced 5-$HT_{1B/1D}$/5-HT_{2A} selectivity.

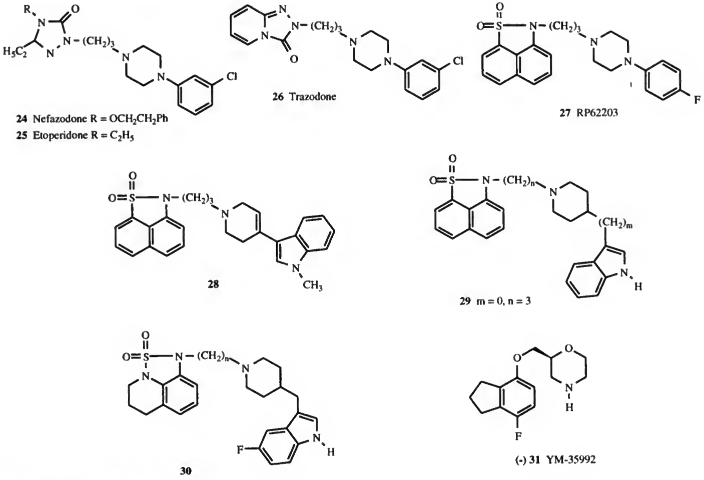
Although formerly reported as a selective 5-$HT_{1B/1D}$ receptor antagonist, GR127935 turned out to be a partial agonist [85]. Its failure to increase brain 5-HT release *in vivo* has been related to this intrinsic activity. SAR studies on the basic side-chain have demonstrated the importance of the spatial orientation of this basic amino residue when designing 5-HT_{1B} receptor antagonists [89]. Thus, the derivative **17** with the (dimethylamino)ethoxy side-chain is a partial agonist, while the (dimethylamino)propyl derivative **18** has been characterized as an inverse agonist.

The benzanilide **19**, is a neutral, potent, and selective h5-HT_{1B} receptor antagonist; its selectivity for the h5-HT_{1B} as compared to the h5-HT_{1D} receptor is remarkable (K_i (h5-HT_{1B}) = 12 nM; K_i (h5-HT_{1D}) = 700 nM [89]). Compounds **19-22** have similar affinitie for the h5-HT_{1B} receptor, but different intrinsic activities. Derivatives **19** and **20** are neutral antagonists, while **21** and **22** are partial agonists. Molecular modeling analyses suggest that, when the common triaryl moiety of the molecules are overlapped, the neutral antagonists and the partial agonists differ by a near-orthogonal orientation of the NH^+ projection to the hydrogen-bond acceptor site. The protonated amino group is positioned above the plane of the benzene ring in the antagonists, whereas it lies in the benzene ring plane in the case of the partial agonists [89].

A series of arylpiperazides of the general structure **23** displays selectivity toward the 5$HT_{1B/1D}$ receptors as compared to the 5-HT_{1A} receptor [90]. Their intrinsic activity depends on the nature of the arylpiperazide moiety. The o-tolyl derivative (**23**, Ar = *o*-tolyl) is a neutral h5-HT_{1B} antagonist *in vitro* and *in vivo* (K_i h5-HT_{1A}) = 49.5 nM; K_i (h5-HT_{1B}) = 0.30 nM; K_i (h5-HT_{1D}) = 0.69 nM).

SSRIs/5-HT_2 Antagonists (Fig. (5))

The marketed antidepressant nefazodone (**24**) is a 5-HT reuptake inhibitor and a 5-HT_2 receptor antagonist (K_i (uptake) =



24 Nefazodone R = OCH_2CH_2Ph
25 Etoperidone R = C_2H_5
26 Trazodone
27 RP62203
28
29 m = 0, n = 3
30
(-) **31** YM-35992

Fig. (5). SSRIs/5-HT2A antagonists.

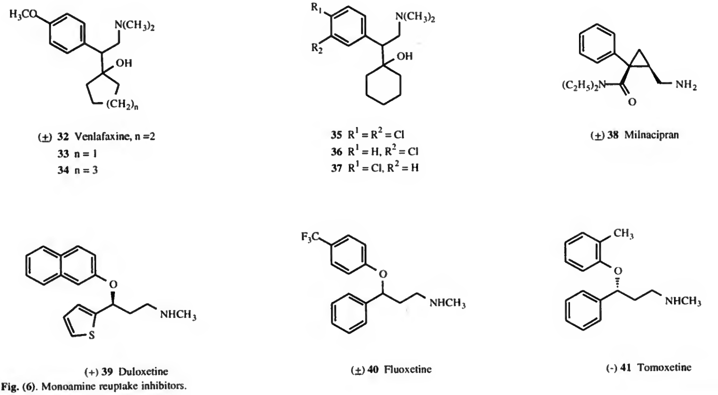
150 nM; K_i (5-HT_2) = 26 nM) [91, 92]. So far, no convincing data for an early onset have been presented. Nefazodone is a m-chlorophenylpiperazine (mCPP) derivative and structurally related to trazodone (**26**) [92] and etoperidone (**25**) [93]. All three compounds exhibit, in addition to their high affinity at the 5-HT_2 receptor, affinities at the α_1-adrenergic and the 5-HT_{1A} receptors [93]. Other *N* heterocycle-propyl substituted mCPP derivatives with 5-HT_{1A} and 5-HT_2 affinity have been described [94]. mCPP, one of the four metabolites of nefazodone, is a 5-HT_{2C} receptor agonist and has been evaluated in an open trial with severely depressed patients [95]. The 5-HT_{2C} receptor is receiving increasing attention as a possible target for novel antidepressants [96].

Other compounds with dual SSRI and 5-HT_2 receptor antagonist properties have been designed [97]. Replacement of the phenylpiperazine moiety in RP62203 (**27**), a potent 5HT_2 receptor antagonist, by 4-(indol-3-yl)-1,2,3,6-tetrahydropyridinyl groups led to compounds with high affinities both for the 5-HT_2 receptor and the 5-HT uptake site. However, substantial affinities for the α_1 and D_2 receptors were also observed. *N*-Methylation of the indole ring did drastically reduce the unwanted α_1 and D_2 receptor affinities without affecting the affinities for the 5-HT_2 receptor and the 5-HT uptake site (**28**, IC_{50} (uptake) = 0.6 nM, IC_{50} (5-HT_2) = 1.3 nM, IC_{50} (α_1, D_2) > 100 nM). Replacement of the tetrahydropyridine ring by a piperidine ring to give **29** improved the 5HT_2 affinity, but gave only moderate affinity for the 5-HT uptake site (IC_{50} (uptake) = 11 nM, IC_{50} (5-HT_2) = 0.05 nM). In the piperidine series, the most selective compounds towards the 5-HT_2 receptor and the 5-HT uptake site as compared to the α_1 and D_2 receptors were obtained with an ethylene chain between the naphthalenesultam ring and the piperidine nitrogen (n=2) and a methylene linker between the piperidine ring and the indole moiety (m=1). Increasing the sum of the two alkyl chains to four, i.e., n=2 and m=2 or n=3 and m=1 (Fig. (**5**), structure **29**), was detrimental to the 5-HT_2 activity while the affinity for the uptake site was retained. Substitution of the naphthalenesultam moiety by a tetrahydroquinoline derivative gave the orally active SSRI/5-HT_2 antagonist 30 (IC_{50} (5-HT uptake) = 1.2 nM, IC_{50} (5-HT_2) = 30 nM).

The 2-(indanyloxymethyl)morpholine YM-35992 (**31**; (*S*)-isomer) is a selective serotonin reuptake inhibitor with 5-HT_{2A} receptor antagonist activity (K_i (uptake) = 21 nM, K_i (5-HT_{2A}) = 86 nM, K_i (5-HT_{2C}) = 680 nM [98]. This compound is in phase I clinical trials.

Specific Serotonin and Noradrenaline Reuptake Inhibitors (SNRIs) (Fig. (6)

Specific serotonin and noradrenaline reuptake inhibitors (SNRIs) have been designed to combine the advantages of TCAs and SSRIs. Like many TCAs, they are dual action drugs, inhibiting both NA and 5-HT uptake and are, thus, expected to have higher efficacy than the SSRIs, especially in severe forms of depression. On the other hand, as the SSRIs, they lack the direct interactions with receptors, notably the cholinergic muscarinic, α_1 adrenergic, and histamine H_1 receptors, which are responsible for the major side-effects of TCAs. Three of the newer compounds with this profile are venlafaxine (marketed), milnacipran (discontinued, phase III), and duloxetine (discontinued, phase II). The marketed drug venlafaxine has been claimed to have a fast onset of action [99]. Using three different statistical methodologies, Derivan *et al.* [30] found venlafaxine to have significant effects early in the course of therapy on or before day 7 of treatment. In all studies an aggressive regimen with a rapid escalation of the dose has been employed, which is not achievable in older antidepressants. As the authors note: "Venlafaxine may be an inherently fast-acting molecule in depressed patients or it may simply be such a well- tolerated drug that sufficient amounts can be given early in the course of the



(±) **32** Venlafaxine, n =2
33 n = 1
34 n = 3

35 $R^1 = R^2 = Cl$
36 $R^1 = H$, $R^2 = Cl$
37 $R^1 = Cl$, $R^2 = H$

(±) **38** Milnacipran

(+) **39** Duloxetine

(±) **40** Fluoxetine

(-) **41** Tomoxetine

Fig. (6). Monoamine reuptake inhibitors.

treatment to induce a relatively rapid response" [30]. From preclinical potency data it is not understandable why venlafaxine should have an earlier onset than paroxetine [100].

Venlafaxine (±**32**; IC_{50} (NA uptake) = 640 nM; IC_{50} (5-HT uptake) = 210 nM) is a less potent analog of **35** (IC_{50} (NA uptake) = 70 nM; IC_{50} (5-HT uptake) = 80 nM), which was abandoned after a positive finding in the Ames test [101]. The 3- and 4-chloro analogs **36** (IC_{50} (NA uptake) = 160 nM; IC_{50} (5-HT uptake) = 320 nM) and **37** (IC_{50} (NA uptake) 300 nM; IC_{50} (5-HT uptake) = 180 nM) were better NA uptake inhibitors *in vitro* than venlafaxine (**32**). The (*S*)-isomer of **32** proved to be a relatively 5- HT selective uptake inhibitor, while the (*R*)-isomer matched the profile of the racemate. The *in vitro* activities of **32**, **33** (IC_{50} (NA uptake) = 5800 nM; IC_{50} (5-HT uptake) = 400 nM), and **34** (IC_{50} (NA uptake) = 2070 nM; IC_{50} (5-HT uptake) = 240 nM) demonstrate the dependence of the NA uptake inhibitory activity on the cycloalkanol ring size; a maximal inhibitory effect was observed with the six-membered ring compound **32**, while 5-HT uptake inhibition was less affected by the ring size. The *O*-demethylated derivative of **32**, which is the major metabolite of venlafaxine in humans [102], is six-fold more potent for 5-HT uptake than for NA uptake (IC_{50} (NA uptake) = 1160 nM; IC_{50} (5-HT uptake) = 180 nM).

Milnacipran (±**38**) is another mixed reuptake inhibitor of NA and 5-HT with moderate *in vitro* and *in vivo* potency (IC_{50} (NA uptake) = 100 nM; IC_{50} (5-HT uptake) = 203 nM) [103]. Clinical trials have shown that the response rate with milnacipran is similar to that with the TCAs, but with a far more benign side-effect profile. No difference in onset of action has been found between milnacipran and TCAs or SSRIs.

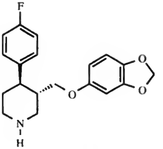
In contrast to other SNRIs, milnacipran is a better NA than 5-HT reuptake inhibitor. The structural features critical for antidepressant activity in this aminomethylcycloprane series are the primary amino group, a tertiary carboxamide function, and preferably an unsubstituted phenyl ring in the α-position of the carboxamide [104]. The (Z) configuration is necessary for activity.

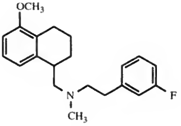
Duloxetine (+**39**) is *in vitro* the most potent SNRI and comes closest to the ideal balance of 5-HT and NA uptake inhibition (IC_{50} (NA uptake) = 7 nM; IC_{50} (5-MT uptake) = 2.6 nM [105]).

It belongs to the aryloxypropanamine class of monoamine reuptake inhibitors, of which the SSRI fluoxetine (**40**) (IC_{50} (NA uptake) = 517 nM; IC_{50} (5-HT uptake) = 22 nM [105]) is the archetype [106]. The same structural class contains tomoxetine (**41**, (*R*)-isomer), which is a selective NA uptake inhibitor (IC_{50} (NA uptake) = 1.9 nM; IC_{50} (5-HT upta 99 nM [105]). Replacement of the phenoxy group by a 1-naphthoxy group led to dual NA/5-HT reuptake inhibitors, such as **39**.

α_2-AdrenoceptorAntagonists/SSRIs (Fig. (7))

Compounus that combine α_2-adrenoceptor antagonism with 5-HT reuptake inhibition were designed by incorporating structural features of the SSRI paroxetine ((3*S*, 4*R*)-**42**) into the α_2-adrenoceptor antagonist **43** (K_i (α_2) = 3.2 nM, IC_{50} (uptake) = 160 nM) that was found by screening [107]. This led to compound **44** (K_i (α_2) = 6.7 nM, IC_{50} (uptake) = 21 nM). In general, deviation from the 5-methoxy substitution at the tetralin ring resulted in a loss of activity at inhibiting 5-HT uptake, while α_2 binding was only moderately affected. The *R*-configuration at the one stereocenter is required for both α_2-receptor and at 5-HT uptake site affinities (*R*)-**44**: K_i (α_2) = 1. 7 nM, IC_{50} (uptake) = 12.7 nM; (*S*)-**44**: K_i (α_2) = 88.6 nM, IC_{50} (uptake) = 1310 nM). *N*-Methyl substitution appears to be essential as the corresponding secondary amine (racemate) displayed poor affinity in both assays (K_i (α_2) = 88.2 nM, IC_{50} (uptake) = 414 nM). By contrast, a notable degree of structural variety is permitted in the arylethyl moiety; a large number of bicyclic substructures is tolerated without significant loss of affinity at the α_2-receptor nor at the 5-HT uptake site. No


(-) **42**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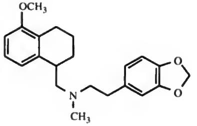
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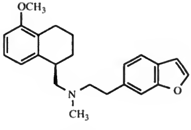
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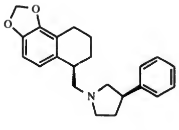

(±) **46** ABT-200

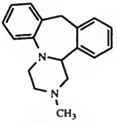
Fig. (7). α_2-Adrenoceptor antagonists/SSRIs.

correlation of SAR data to α_2-antagonist activity is given [107]. Within this series of compounds, A 80426 ((*R*)-**45**) showed excellent affinities at the desired sites (K_i (α_2) = 2.0 nM, IC_{50} (uptake) = 13.1 nM) and excellent selectivity for the 5-HT uptake site *vs.* the noradrenaline and dopamine uptake sites (66- and 224-fold, respectively) [108, 109]. The only other significant affinities were found at the D_2 and 5-HT_2 receptors (52 and 144 nM, respectively). Unfortunately, A 80426 was not an effective α_2-adrenoceptor antagonist *in vivo* and lacked activity in some behavioral models of depression [110].

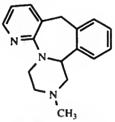
The racemate **46** is an α_2-antagonist with moderate NA uptake inhibitory activity [111]. The α_2-antagonist activity resides mostly in the (*R,R*)-enantiomer (K_i (α_2) = 0.4 nM; IC_{50} (NA uptake) = 3110 nM), while the NA uptake inhibitory activity is mostly associated with the *S,S*-enantiomer (K_i (α_2) = 2.1 nM; IC_{50} (NA uptake) = 636 nM). No efficacy was found with ABT- 200 in a clinical study [112] and its development has been discontinued.

Noradrenergic and Specific Serotonergic Antidepressants (NaSSA; (Fig. (8))

Regulation of noradrenaline release in the human brain involves release-inhibiting, presynaptically located α_2-adrenoceptors (auto-receptors). α_2-adrenoceptors (heteroreceptors) exist also on 5-HT terminals, where their blockade facilitates 5-HT release. Selective α_2-inhibitors, like idazoxan and fluparoxan, have been developed, but the published data suggest antidepressant action similar to that of amitryptiline "with even some hint of a fast onset of action "[5].



47 Mianserin



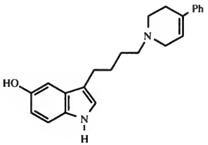
47 Mirtazapine

Fig. (8). Mianserin and mirtazapine.

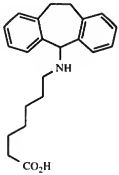
The tetracyclic derivative mianserin (**47**) has been available for two decades; it combines α_2-adrenoceptor antagonism with modest NA reuptake inhibition; however, it also has significant α-adrenoceptor blocking properties [113]. Mirtazapine (**48**) was designed within this series of tetracyclic compounds with the intention to extend the pharmacological profile of mianserin [9, 114, 115]. Mirtazapine is designated as a noradrenergic and specific serotonergic antidepressant (NaSSA), as its α_2-antagonsim accounts for both its pro-adrenergic and pro-serotonergic properties (K_i (α_2) = 100 nM). At the same time, the 5$HT_{2\ (A\ and\ C)}$ and 5-HT_3 receptors are directly blocked by mirtazapine (K_i (5-HT_{2A}) = 10 nM), which leads to a reduction of serotonergic side-effects associated with non-selective 5-HT receptor stimulation by SSRIs [7].

D_2/D_3 Agonists[3] *(Fig. (9))*

Chronic treatment with antidepressant drugs irrespective of the primary neurochemical action of the drug causes a sensitization of D2/D3 receptor mediated responses in the mesolimbic systems [117, 118]. It has been suggested that this might represent a final common pathway through which chronically administered antidepressants influence behavioral output systems, as low doses of D2/D3-receptor antagonists reverse the actions of antidepressants [119, 120].



49 Roxindole



50 Amineptine

Fig. (9). dopaminergic drugs.

A rapid onset of action could be expected for dopamine agonists, as they act downstream, bypassing the adaptive changes in the 5-HT circuitry, which are thought to be responsible for the slow onset. Yet, similar adaptive changes within the dopaminergic system may antagonize a rapid onset.

The relevance of dopamine in the pathophysiology of depression has been both questioned and supported recently [121]. While many issues remain to be determined (*e.g.*, the only modest antidepressive effect of L-Dopa and MAO-B inhibitors; the function of the different dopamine receptor subtypes), new specific D2/D3 dopamine agonists like pramipexole [122] and ropinirole [123] might help to clarify these hypotheses.

A rapid onset of action has been claimed in recent clinical trials for several drugs acting at the dopamine system, including roxindole (**49**) [124, 125], and amineptine (**50**) [29, 126, 127]. Roxindole is a D2/D3-agonist, 5-HT uptake inhibitor, and 5-HT_{1A} agonist (IC_{50} (D2) = 0.5 nM, IC_{50} (5-HT_{1A}) = 0.9 nM, IC_{50} (5-HT uptake) = 0.2 nM) [128]. Amineptine is an atypical tricyclic monoamine reuptake blocker, having a 7-aminoheptanoic acid side chain. It has a distinct pharmacological action in its ability to inhibit both the dopamine uptake and increase dopamine release [129]. Amineptine is available in only a few markets, probably, due to its potential for abuse.

Concepts Beyond the Monoamine Theory of Depression

Although the monoamine theory of depression postulates that the symptoms of the illness arise as a consequence of a defect in the availability of biogenic amines, neither of these neurotransmitters seems to be essential to maintain normal mood nor does a single neurotransmitter account for all symptoms of the depressive syndrome [27]. Consequently, abnormalities in other neurobiological systems underlie depressive symptoms. Nevertheless, the biogenic amines have a major modulatory role on neurobiological systems involved in the recovery from depression.

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[3] Recently, subtypes (D2; D3; D4) of the former described dopamine D2 receptor have been characterized [116].

Several currently discussed systems, which might be involved in the neurobiology of depression or in the recovery from depressive symptoms, such as adenosine-receptors [130, 131], σ-receptors [132], GABA- receptors [133] and NMDA-receptors [134], have not been considered in detail in this article; available data concerning their role in modifying onset of action are scarce or inconclusive.

Moreover, hormones play a critical role in maintaining normal mood [135, 136]. Particularly, disturbances in the hypothalamic-pituitary-thyroid axis and the hypothalamicpituitary-adrenocortical axis are common in patients with affective disorders [137]. There is evidence, that *l*-trliodothyronine (T3) can accelerate the onset of imipramine's antidepressant effects [138], and injection of thyroid stimulating hormone (TSH) in depressed women can cause a quick but brief and partial antidepressant response [139]. However, no controlled clinical studies are available for these observations and the limited extent of clinical data precludes drawing conclusions in regard to an accelerating effect of action.

Additional targets exist beyond the receptor level, particularly at the level of signaling transduction and gene expression. Several lines of evidence indicate that neuroadaptive changes, which seem to be important for mediating the clinical effects of antidepressant drugs, occur at a level beyond the receptor involving signaling transduction pathways [10, 140, 141]. The progress in molecular biology should permit the identification of susceptibility genes [142] and a more complete characterization of changes in gene expression [143, 144] that are involved in the pathophysiology of depression. Unraveling the functional alterations of components of the intracellular signaling transduction pathways and the changes in differentially expressed genes may reveal a host of new targets for drug discovery. Given their mode of action, such targets should theoretically result in a more immediate impact and, thereby, possibly shorten the delayed onset of action that plagues currently available antidepressant drugs.

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Oleamide: An Endogenous Sleep-inducing Lipid and Prototypical Member of a New Class of Biological Signaling Molecules

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Abstract: Oleamide is an endogenous fatty acid primary amide that accumulates in the cerebrospinal fluid under conditions of sleep deprivation and induces physiological sleep in animals. A review covering its discovery, its implications, and the emerging biology surrounding its discovery is presented. Consistent with its role as a prototypical member of a new class of biological signaling molecules, enzymatic regulation of endogenous concentrations of oleamide have been characterized or proposed. Fatty acid amide hydrolase (FAAH) is an integral membrane protein that degrades oleamide and potent inhibitors with physiological sleep-inducing properties have been disclosed. The characterization, cloning, and neuronal distribution of FAAH have been detailed and the enzyme was found to possess the ability to hydrolyze a range of fatty acid amides including anandamide which serves as the endogenous ligand for the cannabinoid receptor. An additional endogenous substance with REM sleep-inducing properties, 2-octyl γ-bromoacetoacetate, was characterized as a potent FAAH inhibitor. Oleamide has been shown to modulate serotonergic neurotransmission and inhibit intercellular gap junction communication and detailed studies of its well defined and selective structural features required for activity have been disclosed.

Introduction

Although it is easy to appreciate the extent of existing sleep disorders and the discomfort experienced following a restless night of sleep, remarkably little is known about the physiological control and mechanism of sleep. This is surprising since we spend approximately 1/3 of our lifetime (>25 years) sleeping. In addition to gaining an appreciation of the full role of sleep, an obvious benefit to understanding its physiological basis would be the identification of therapeutic targets useful for the treatment of sleep disorders. Approximately 1/3 of all U.S. adults experience intermittent insomnia and it is estimated that >50 million Americans alone suffer from chronic or intermittent sleep disorders [1]. Typically, these are not treated or averted leading to decreased quality of life, lower personal productivity, and increased public safety hazards [1]. Current treatments typically enlist sedative or hypnotic drugs that do not induce physiological sleep [1-3]. Rather, they act as general CNS depressants that do induce slumber, but do not fulfill the full role of physiological sleep. It is less restful than physiological sleep and is accompanied by a range of side effects including an impact on memory and orientation. Sustained treatment suffers from rebound sleep where the body reacts to drug discontinuance by requiring additional physiological sleep to recoup functions lost during drug-induced sleep. One of the most important characteristics of physiological sleep, the choice to sleep and the ability to wake to the sound of a disturbance, is disrupted. One important function, the reprocessing of daily short term memory to long term memory, is impaired. In addition, placement of typical hypnotic or sedative sleep aids in the public has suffered from abuse, most notably suicide derived from an overdose of the general CNS depressants. Many of these shortcomings may be addressed once the underlying basis for the induction and control of physiological sleep is better understood.

A beautiful series of studies have provided a basis for such investigations. At the beginning of this century, two groups independently showed that endogenous substances can induce sleep in alert, fresh animals. Both Ishimori [4] in Japan and Legendre and Piéron [5] in France demonstrated that brain tissue, cerebrospinal fluid (CSF), or blood plasma taken from sleep-deprived dogs and administered to fresh, alert dogs resulted in the induction of behavioral sleep. At the same time, dogs receiving similar treatments taken from alert dogs remained unaffected. Although unpopular at the time [6-10], Ivy and Schnedorf [11,12] reestablished the presence of endogenous sleep inducing factors in 1939 again in descriptive behavioral studies.

With the introduction of more objective techniques enlisting electroencephalogram (EEG) monitoring, the search for sleep substances reemerged in the 1960s. Kornmüller *et al.* were the first to demonstrate the blood-borne transfer of EEG sleep from one cat to another [13], Monnier made similar observations with rabbits [14], and Pappenheimer demonstrated that the CSF transfer was species independent being capable of transfer between mice, rats, rabbits, dogs, cats, and goats [15,16]. CSF fractionation studies by Pappenheimer [16] implicated an active substance with a molecular weight less than 500 g/mol. Subsequent demonstration of its peptidase sensitivity seemingly implicated a peptide [17]. Due largely to the prohibitive required volumes of collectible CSF, the structure of this substance (Factor S) was never unambiguously established.

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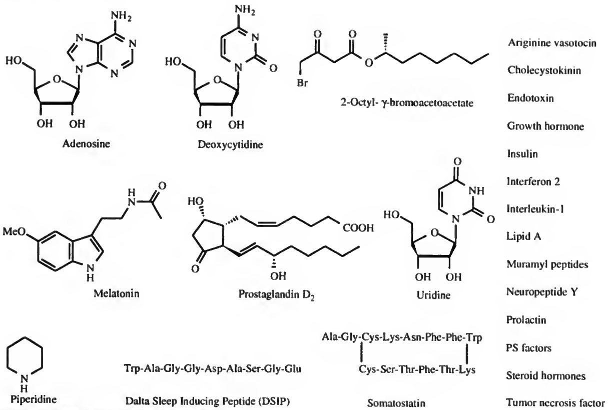


Fig. (1). Reported and putative sleep-promoting substances.

Throughout the intervening years, a number of sleep-inducing substances have been identified, Fig. (**1**) [2,3]. This includes Monnier's characterization of delta-sleep-inducing peptide (DSIP) in the blood of sleeping rabbits electrically stimulated in the thalamic "sleep center" [18], Uchizono's identification of uridine as one of the components of the sleep-promoting substance (SPS) found in a brainstem extract which increased slow wave sleep (SWS) and rapid eye movement (REM) sleep [19], Yanagisawa's identification of 2-octyl γ-bromoacetoacetate as an endogenous human CSF constituent that induces REM sleep in rats [20], Ueno's characterization of prostaglandin D_2 as a SWS-inducer [21] and McCarley's characterization of adenosine as an endogenous regulator of sleep [22]. None are universally accepted as a single causative agent of sleep, not many of the molecules illustrated in Fig. (**1**) are found in the brain or CSF, and even fewer meet the Pappenheimer structural and fractionation criterion.

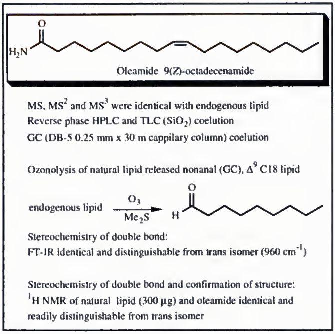
Isolation, Structure Determination, and Characterization of Oleamide

Reverse-phase HPLC fractionation comparisons of the CSF from normal and sleep-deprived cats led to the identification of a substance that accumulated under conditions of sleep deprivation [23]. Upon resting, CSF from the same animals lacked the substance found when sleep deprived. [23]. FABHRMS provided a best fit molecular formula of $C_{18}H_{35}NO$ and MS^2/MS^3 revealed a lipid fragmentation pattern following sequential losses of NH_3 and H_2O [23]. Given the trace quantities of material found in the CSF that was generated only upon the induced conditions of sleep deprivation, efforts to isolate sufficient quantities for characterization and structure identification proved unrealistic. With this information in hand (Fig. (**2**)) and given the apparent simplicity of the molecule, a large number of candidate lipid structures incorporating the molecular formula and correct degrees of unsaturation were prepared by chemical synthesis and correlated with the endogenous substance [24,25]. Oleamide (**1**) proved identical with the authentic material and upon recognition of its characteristics and solubility properties, sufficient amounts (300-400 μg) of the pure endogenous lipid were isolated to permit an unambiguous correlation [24,25], Fig. (**3**).

1) Reverse Phase HPLC detection:
   CH_3CN-H_2O (0.1% TFA) in CSF of sleep-deprived cats
2) HRFABMS *m/z* 304.2614 (M^+ + Na): Mol. Formula = $C_{18}H_{35}NO$
   tow degrees of unsaturation, odd number of nitrogens
   fragmentation of *m/z* 265 (M^+ - NH_3)
   subsequent fragmentation of *m/z* 247 (M^+ - NH_3 - H_2O)
   lipid fragmentation pattern
3) ≤ 100 μg finally isolated but impure
   UV: may be UV absorbance at 215 nm
   NMR: lipid structure, conjugated or unconjugated olefin(s)?
   continued focus on isolation unrewarding
   trace quantities available only upon sleep deprivation
   hampered by loss of product during isolation (stability?)

Fig. (2). Characteristics of the endogenous lipid.

Synthetic oleamide was found to induce sleep in rats, mice, and cats in a dose dependent manner [24]. As in physiological sleep, the animals still maintained the ability to respond to auditory stimuli with an orienting reflex and a sustained attention toward the source. Intraventricular injection of



MS, MS^2 and MS^3 were identical with endogenous lipid
Reverse phase HPLC and TLC (SiO_2) coelution
GC (DB-5 0.25 mm x 30 m cappilary column) coelution

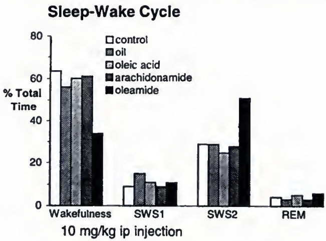
Ozonolysis of natural lipid released nonanal (GC), Δ^9 C18 lipid

Stereochemistry of double bond:
FT-IR identical and distinguishable from trans isomer (960 cm^{-1})

Stereochemistry of double bond and confirmation of structure:
^1H NMR of natural lipid (300 μg) and oleamide identical and readily distinguishable from trans isomer

Fig. (3). Key elements of structure identification.

oleamide (2.8 μg, 10 nmol) induced electrophysiologically monitored sleep analogous to its ip administration (5 or 10 mg/kg) indicating it acts directly in the brain at a dose comparable with other known effector molecules (Fig. (1)). Polysomnographic studies revealed an increase in the total time of slow wave sleep (SWS) as well as in the mean duration of SWS, especially SWS2, at the expense of waking, Fig. (4). Distribution of REM sleep does not seem to be altered. The examination of a number of close structural analogs revealed that this effect is specific for oleamide. The corresponding trans isomer, (9*E*)-octadecenamide, exhibited weaker effects and only at higher doses, (8*Z*)-octadecenamide, (11*Z*)-octadecenamide and (13*Z*)-docosenamide were even less effective, while the saturated counterpart stearamide, as well as arachidonamide and oleic acid were ineffective [24]. (13*Z*)-Docosenamide was also detected in rat and human CSF [24], but its concentration did not oscillate with sleep-wake cycles. Unlike the behavior upon treatment with sedatives or hypnotics but consistent with the choice to sleep naturally, acclimation of the animals to their treatment environment, (*e.g.* cages) and their pre-/post-treatment handling have been found to impact the reproducibility of oleamide-induced sleep [26].



- oleamide induces sleep in a dose dependent manner: duration of effect
- maintain orienting reflex toward noise
- structurally specific for oleamide
  $18{:}1^9$(trans) much weaker but similar
  $18{:}1^8$ and $18{:}1^{11}$ even weaker (not sleeping)
  $20{:}1^{11}$ and $22{:}1^{13}$ even weaker (not sleeping)
  18:0, oleic acid, arachidonamide inactive
- lowers body temperature
  dose dependent manner
  magnitude and duration of effect

Fig. (4). Oleamide induces physiological sleep in rats.

Characteristic of physiological sleep, oleamide administration also lowers the body temperature (hypothermia) in a pronounced and dose dependent fashion [26].

These studies place oleamide among a small but growing class of endogenous or naturally occurring fatty acid amides. Prior to our identification of oleamide in human CSF and the CSF of sleep-deprived cats, the primary amides of oleic ($18{:}1^9$), palmitic (16:0), palmitoleic ($16{:}1^9$), claidic ($18{:}\ 1^{9trans}$), and linoleic ($18{:}2^{9,12}$) acids were identified in human plasma [27] before physiological roles were established. Erucamide, (13*Z*)-docosenamide or $22{:}1^{13}$, has been identified as the major angiogenic component in bovine mesenterial lipids stimulating new blood vessel formation and growth [28]. The endogenous presence of fatty acid ethanolamides and their potential roles have been reviewed [29] and palmityl, oleyl, and arachidonyl ethanolamides have been found to be an anti-inflammatory agent [30], a ceramidase inhibitor blocking the formation of sphingosine [31], and an endogenous ligand for the cannabinoid receptor [32], respectively. In the continued examination of oleamide, including studies of its synthesis, storage, release, degradation, regulation, and potential sites of action, it is clear the fatty acid amides are emerging as a new class of important biological signaling molecules. Probably the best understood feature of this class is their degradative metabolism by the newly characterized enzyme fatty acid amide hydrolase (FAAH) [24,33-35]. Little is known about the endogenous synthesis, storage, or release of oleamide [36-38], and insights into its site(s) of action are only now beginning to emerge, Fig. (5). Potent inhibitors of FAAH with sleep inducing properties have been disclosed [39] and the endogenous (human CSF) sleep inducing molecule 2-octyl γ-bromoacetoacetate (Fig. (1)) has been found to be a potent, reversible inhibitor of FAAH [40]. Oleamide has been shown to modulate serotonin receptor responses potentiating the 5-HT_{2A}, 5-HT_{2C}, and 5-HT_{1A} receptor subtypes [41,42] but inhibiting 5-HT_7 [43] in a structurally specific manner. Similarly, it inhibits rat glial gap junction communication [44-46] and does so in a manner that deconvolutes the previously indistinguishable intercellular transfer of Ca^{+2} and small molecules. In the instance where this was examined, the endogenous concentration of oleamide was found to be approximately 100x that of anandamide [38]. Thus, unlike anandamide whose low concentrations have raised reservations over its endogenous role, oleamide is present in concentrations to have a physiological role. Like anandamide, oleamide has also been shown to inhibit lymphocyte proliferation [47] without cell toxicity and oleamide causes

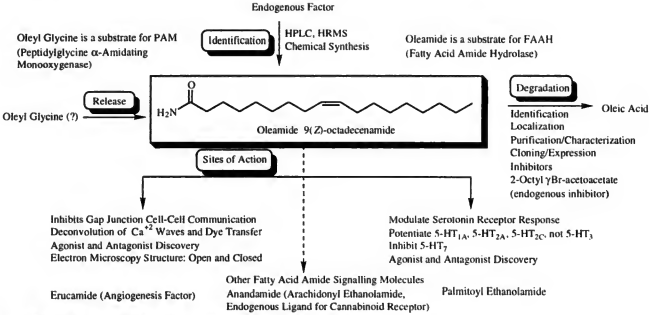
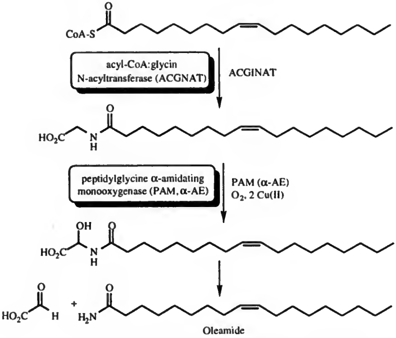

Endogenous Factor
Identification
HPLC, HRMS
Chemical Synthesis
Oleyl Glycine is a substrate for PAM
(Peptidylglycine α-Amidating Monooxygenase)
Oleamide is a substrate for FAAH
(Fatty Acid Amide Hydrolase)
Release
Oleyl Glycine (?)
O
H2N
Oleamide 9(Z)-octadecenamide
Degradation
Oleic Acid
Identification
Localization
Purification/Characterization
Cloning/Expression
Inhibitors
2-Octyl γBr-acetoacetate
(endogenous inhibitor)
Sites of Action
Inhibits Gap Junction Cell-Cell Communication
Deconvolution of Ca+2 Waves and Dye Transfer
Agonist and Antagonist Discovery
Electron Microscopy Structure: Open and Closed
Modulate Serotonin Receptor Response
Potentiate 5-HT1A, 5-HT2A, 5-HT2C, not 5-HT3
Inhibit 5-HT7
Agonist and Antagonist Discovery
Other Fatty Acid Amide Signalling Molecules
Erucamide (Angiogenesis Factor)
Anandamide (Arachidonyl Ethanolamide, Endogenous Ligand for Cannabinoid Receptor)
Palmitoyl Ethanolamide

Fig. (5). Advances in the chemistry and biology of oleamide.

similar pharmacological effects as anandamide in the tetrad of cannabinoid assessments albeit without cannabinoid (CB1) receptor binding [48].

Synthesis, Storage, and Release

Little is presently known about the synthesis, storage, or release of oleamide. Merkler has shown that N-oleyl glycine, enzymatically assembled by acyl-CoA:glycine N-transferase catalyzed condensation of glycine with oleyl-CoA, is an effective substrate for peptidylglycine α-amidating monooxygenase (PAM, αAE) which α-hydroxylates the glycine α-position, Fig. (6) [36]. The resulting N-acyl hemiaminal breaks down with release of oleamide. It is not known whether N-oleyl glycine is an effective precursor of endogenous oleamide or whether related N-oleyl Gly peptides may serve as precursors,


CoA-S
O
acyl-CoA:glycin
N-acyltransferase (ACGNAT)
ACGINAT
HO2C
N
H
peptidylglycine α-amidating
monooxygenase (PAM, α-AE)
PAM (α-AE)
O2, 2 Cu(II)
OH
HO2C
H
+
H2N
Oleamide

	D-Tyr-Val-Gly	N-Myristoyl Gly	N-Oleyl Gly
K_M (μM)	47	127	290
V_M (S^{-1})	19.8	29.4	23.1
V_M/K_M	4.21	2.31	0.80
K_i(mM)	1.0	0.7	0.04

- analogous to release of primary amide peptide chemical messengers and hormones

Fig. (6). Proposed synthesis, storage and release.

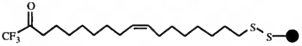
but its close relationship to the analogous storage and release of neuroactive peptides terminating in a primary amide presents an attractive proposal. In complementary studies, it has been shown that oleamide is produced from oleic acid directly in mouse neuroblastoma cells at high levels (55 pmol/10^7 cells) and that it is unlikely to occur through direct FAAH catalyzed condensation with ammonia [38] although an earlier study had shown that enzymatic activity in rat brain microsomes catalyzed this transformation [37].

Degradation and Regulation

In contrast to its synthesis, storage, and release, the degradation of oleamide is well understood. At the time of its identification, membrane bound enzymatic activity was detected that resulted in the hydrolysis of oleamide to oleic acid and ammonia [24]. This activity was inhibited by both phenylmethylsulfonyl fluoride (PMSF) and $HgCl_2$ but was not inhibited by EDTA implicating a serine or cysteine protease versus metalloprotease. The purification and characterization of the enzymatic activity was accomplished enlisting an inhibitor-bound affinity chromatography, Fig. (7) [33].

$$\begin{matrix}\text{Oleamide} \\ \text{Anandamide}\end{matrix} \xrightarrow[\text{Inactivation}]{\text{FAAH}} \begin{matrix}\text{Oleic Acid} \\ \text{Arachidonic Acid}\end{matrix}$$

- memberane-bound activity
- Integrally-bound membrane protein
- Not inhibited by EDTA (not metalloenzyme)
- Inhibited by $PhCH_2SO_2F$ (PMSF) and $HgCl_2$
- Inhibitor-bound affinity chromatography purification



- Tissue distribution (++ in brain and liver)
- Rat, mouse and human FAAH cloned
- Rat, mousn and human FAAH characterized
  single-copy gene: 579 aa, 100% 91% and 84% homology
  transmembrane domain:
  9-ALPGASGVALACCFVAAAVAL-29
  conserved amidase consensus sequence:
  215-GGSSGGEGALIGSGGSPLGLGTDIGGSIRFPS-246
  conserved SH3 domain: 310-PPLPFR-315
- CNS distribution (neurons, gilal cells
  Highest: hippocampus
  Modest: thalamus, cerebellum, hypothalamus, cortex, olfactory bulb
  Lowest: striatium, pituitary
- CNS overlap with regions associated with sleep, motivational and emotional states, 5-HT and CB1 receptor localization
- Selectivity

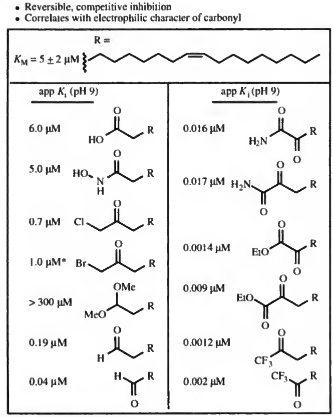
	human	rat	fatty acid amide
rel. rate of hydrolysis	1.0	1.0	Anandamide
	0.70	0.73	$18{:}1^9$,Oleamide
	0.65	0.24	$14{:}1^9$,Myristoleamide
	0.33	0.10	$16{:}1^9$,Palmitoleamide
	0.06	0.06	18:0, Stearamide

Fig. (7). FAAH characterization.

The affinity purified rat liver oleamide hydrolase was subjected to tryptic digestion and the resulting peptides were separated by HPLC and sequenced. This protein sequence information in turn facilitated the cloning of the cDNA encoding this enzyme, which was transiently expressed in COS-7 cells to demonstrate conclusively that an enzyme responsible for the hydrolysis of oleamide had been isolated [33]. Additionally, the expressed oleamide hydrolase also degraded several other fatty acid amides, including anandamide, indicating that the enzyme may serve as an *in vivo* mechanism for the inactivation of the fatty acid amide family of neuromodulatory molecules. In recognition of the plurality of fatty acid amides which oleamide hydrolase can accept as substrates, we have renamed the enzyme as fatty acid amide hydrolase, or FAAH. Database searches with the FAAH protein sequence revealed that the enzyme was the first identified mammalian member of a previously defined amidase family [49], whose members all share a common signature sequence. Further analysis of the FAAH protein identified a putative transmembrane domain from amino acids 10-29, supporting biochemical data that FAAH is an integral membrane protein. A class II SH3binding domain sequence PPLXR [50], was also found in the FAAH sequence, suggesting that other signaling proteins may interact with FAAH to regulate its activity [51] or subcellular localization [52]. Northern analysis identified FAAH mRNA as most abundant in liver and brain, with low levels of expression in several other tissues [33]. No FAAH message was detected in skeletal muscle or heart. Interestingly, this profile for the rat FAAH mRNA matches nearly exactly the tissue distribution for FAAH enzyme activity [35], providing further evidence that FAAH may act as the primary, if not sole means for fatty acid amide hydrolysis *in vivo*. In this regard, Southern analysis of the FAAH gene proved most consistent with FAAH being derived from a single copy gene [33].

In order to identify further those regions of the FAAH protein critical to the enzyme's function, human and mouse homologs of the rat FAAH were cloned and characterized [34]. The three FAAH enzymes proved highly homologous, sharing greater than 82% identity. The previously discussed amidase signature sequence and SH_3-binding domain sequence were completely conserved in all three enzymes. The human FAAH was shown to possess similar substrate selectivity and inhibitor sensitivity to the rat FAAH, indicating that potent rat FAAH inhibitors will likely also serve as effective disruptors of human FAAH activity.

Recently, the brain distribution of rat FAAH has been characterized in more detail by *in situ* hybridization and Northern analysis [35a]. The rat FAAH message was found to be developmentally regulated in the brain, first appearing weakly at embryonic day 14 and increasing progressively until postnatal day 10 where maximal FAAH expression was maintained until postnatal day 30. FAAH levels were then found to decrease slightly to a stable intermediate expression level in the adult. *In situ* hybridization revealed profound distribution of FAAH mRNA in neuronal cells throughout the brain. The most prominent signals were detected in the neocortex, hippocampal formation, amygdala, and cerebellum. The distribution of FAAH in the CNS suggests that this enzyme is posed to degrade neuromodulatory fatty acid amides at their sites of action, and through doing so, will likely influence the intensity and duration of the behavioral effects evoked by these molecules.



*different assay conditions, IC_{50}

Fig. (8). Representative inhibitors of FAAH.

More recently, both 1- or 2-arachidonoylglycerol [53], also endogenous ligands for the cannabinoid receptors [54-57], as well as methyl arachidonate [53] have been found to be hydrolyzed and inactivated by FAAH.

Fatty Acid Amide Hydrolase (FAAH) Inhibitors

Potent inhibitors of the enzyme responsible for the degradation of oleamide can be expected to potentiate the effects of the endogenous lipid and potentially induce physiological sleep. A series of potent, selective, and reversible, competitive inhibitors (K_i = 13 μM-1 nM) of fatty acid amide hydrolase were disclosed shortly after the discovery of oleamide and FAAH, Fig. (**8**) [39]. The most potent inhibitors possess an electrophilic carbonyl capable of reversible (thio)hemiacetal or (thio)hemiketal formation mimicking the transition state of a serine or cysteine protease catalyzed reaction. The relative potency of the inhibitors follows the expected trends of the electrophilic carbonyls culminating in the tight-binding α-keto esters (1.4 nM) and trifluoromethyl ketones (1.2 nM), Fig. (**9**). Though preliminary, the inhibitor potencies of the α-keto amides which are unlikely to bind as simple hydrates and the nonpotent activity of the corresponding alcohol or ketal/acetal suggest the inhibitors bind as reversible, covalent (thio)hemiketals rather than simple hydrated gemdiols. The position of the electrophilic carbonyl in the inhibitors appears flexible consistent with the enzymes role in degrading multiple fatty acid amide substrates, but the inhibitors did exhibit a prominent sensitivity to the presence of the oleamide *cis* Δ^9 double bond. None of the potential irreversible inhibitors exhibited time-dependent or irreversible enzyme inhibition (up to 15 min) suggesting FAAH may not be a classical cysteine protease [58] and further implicating a serine protease.

Preliminary studies with four FAAH inhibitors revealed sleep-inducing properties in rats for the three more potent inhibitors comparable to the effects observed with oleamide itself, Fig. (**10**) [26]. In the case examined, the trifluoromethyl ketone inhibitor also produced a lowering of body temperature (hypothermia) analogous to oleamide although the effect was

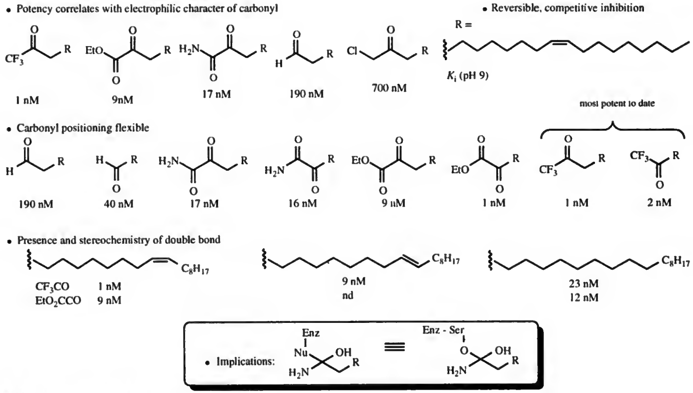
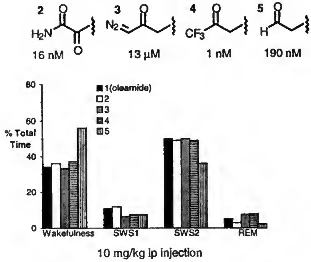


Fig. (9). FAAH inhibitor trends.



- Inhibitors have same effect as oleamide
  induce physiological sleep in rats
  increase SWS2 at the expense of waking
- 4 lowers body temperature (0.5 °C in rats)
  less pronounced than oleamide (2-3 °C)
  slower onset (1 h delay)

Fig. (10). FAAH inhibitors induce physiological sleep.

less pronounced and occurred with a slower onset. These observations suggest, but do not require, that FAAH may be a productive therapeutic target for the development of sleep aids which act by inhibiting oleamide degradation and potentiating the effects of the endogenous lipid. In addition, some such inhibitors including the α-keto amides may be oleamide agonists in their own right and thus possess the dual properties of oleamide agonist/FAAH inhibitor that contribute to their sleep inducing properties.

Also consistent with such effects, ip administration of the FAAH inhibitor phenylmethylsulfonyl fluoride (PMSF) has also been shown to induce hypothermia, immobility, and antinociception [59]. An analogous series of trifluoromethyl ketone, α-keto ester, and α-keto ethanolamide inhibitors [60] were disclosed for anandamide hydrolase which later proved identical to FAAH, palmitylsulfonyl fluoride [61] was found to inhibit the enzyme, and methyl arachidonyl fluorophosphonate has been disclosed to be a potent, irreversible inhibitor [62]. While oleamide and arachidonamide do not appear to interact with the CB1 cannabinoid receptor, the arachidonyl-based inhibitors often display an affinity for the CB1 receptor [60-62]. Ibuprofen and suprofen, nonsteroidal anti-inflammatory drugs, have also been shown to inhibit anandamide hydrolysis at pharmacologically relevant concentrations [63].

In addition to potential therapeutic applications, FAAH inhibitors have proven to be useful in probing the biological role of oleamide and anandamide under cellular conditions where they are normally rapidly degraded [48, 59-62], employed in affinity chromatography for the purification and characterization of the integrally membrane bound enzyme [33], and enlisted in electron microscopy structural studies of the oleamide-induced closed gap junction [64].

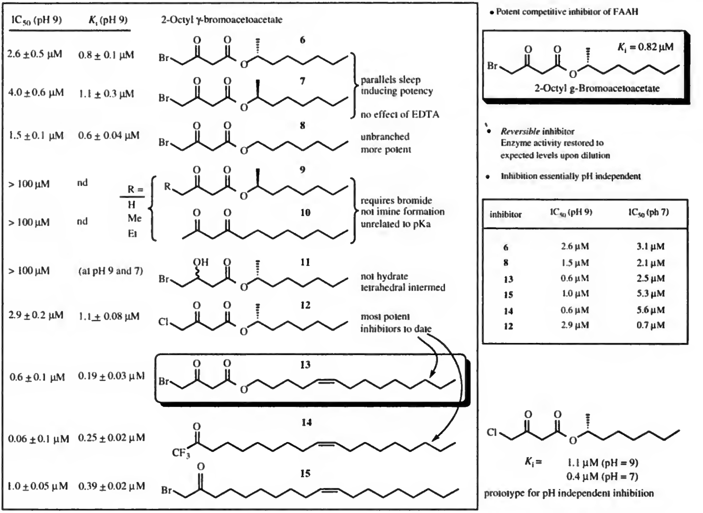
Endogenous 2-Octyl γ-Bromoacetoacetate is a Potent Inhibitor of FAAH

Following early studies that identified an endogenous bromine containing sleep-inducing hormone [65], Yanagisawa was able to isolate and characterize an endogenous REM sleep-inducing compound from human cerebrospinal fluid (40 mg/23 L CSF) [66] and demonstrate that it induced REM sleep in cats (iv @ 0.1 mg/kg) [20]. The compound, 2-octyl γ-bromoacetoacetate, was subsequently isolated from rat cortex, pituitary, and retinal tissues [67], and found to inhibit rat acetylcholinesterase albeit at a modest level (K_i = 32.6 μM) [68]. Given its structural relationship to oleamide, it was examined and found to be a potent, reversible inhibitor of FAAH (K_i = 0.8 μM), nearly as effective as the trifluoromethyl ketone inhibitors [40]. Although initially expected to be an irreversible inhibitor, the kinetics of inhibition at modest time intervals (1 h, pH 7) were those of a reversible competitive inhibitor, Fig. (**11**).

A series of structurally related analogs were examined [40]. Inversion of the C1' configuration did not significantly alter the inhibitor potency while removal of the methyl group increased the potency. Replacement of the bromine with a chloride did not greatly impact the K_i, but its removal or replacement with a methyl or ethyl group as well as reduction of the ketone produced inactive compounds. Analogous β-diketones which possess enolization properties and a p*K*a similar to those of a γ-bromo, β-keto ester were also inactive. Incorporation of the γ-bromo β-keto ester into the oleamide structure provided the exceptionally potent FAAH inhibitor **13** equipotent with the trifluoromethyl ketone inhibitor **14**. Just as interestingly, the inhibition by 2 octyl γ-bromoacetoacetate proved to be independent of pH. When assayed at the physiologically relevant pH 7 rather than pH 9, conditions for optimal enzyme activity, the potency of **6** does not decrease like the behavior of the classical inhibitors and that of the closely related inhibitor **12** actually increased. At pH 7, **6** and **12** surpass the potency of even the trifluoromethyl ketone inhibitor **14** and are the most potent reversible inhibitors disclosed to date. This pH independent character of **6** and its potency at pH 7 underscores the possibility that it could be serving as an endogenous regulator of FAAH activity [40]. In addition to providing a novel template on which to design new protease inhibitors, it may also constitute nature's solution to the design of an electrophilic carbonyl capable of covalent, tight-binding serine protease inhibition.

Serotonin Receptor Modulation

Serotonin receptors have been implicated in anxiety, depression, appetite, thermoregulation as well as sleep and mood regulation and strong links between 5-HT_1, 5-HT_2, and 5-HT_7 and the regulation of sleep have been disclosed [69,70]. Similarly, 5-HT_{1A} agonists have been reported to cause hypothermia [69-71] and similar effects are observed with oleamide. Recent studies have shown that oleamide modulates serotonergic neurotransmission [41-43]. In the first disclosure of such effects, oleamide was shown to potentiate 5-HT_{2C} and 5-HT_{2A} receptor-mediated chloride currents in transfected frog oocytes, but not those elicited by the 5-HT_3 ion-gated channel



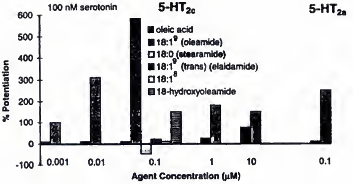
inhibitor	IC_{50} (pH 9)	IC_{50} (ph 7)
6	2.6 μM	3.1 μM
8	1.5 μM	2.1 μM
13	0.6 μM	2.5 μM
15	1.0 μM	5.3 μM
14	0.6 μM	5.6 μM
12	2.9 μM	0.7 μM

- Implications: FAAH is a serine protease 6 released to regulate FAAH activity *in vivo* (pH 7)?

Fig. (11). 2-Octyl γ-bromoacetoacetate: characterization of inhibition of FAAH and new inhibitor design.

receptor or other G protein coupled receptors [41]. This potentiation was greatest for the 5-HT_{2C} receptor subtype where the effect was observed at concentrations as low as 1 nM and was maximal at 100 nM oleamide. Oleamide did not alter the serotonin EC_{50} but instead increased receptor efficacy, Fig. (**12**). An analogous potentiation of the 5-HT_{1A} receptor response has been disclosed and a detailed study of the structural features of oleamide required for potentiation of the 5-HT_{2A} and 5-HT_{1A} response to serotonin (5-HT) has been described [42]. Of the naturally occurring fatty acids, the primary amide of oleic acid (oleamide) is the most effective at potentiating the 5-HT_{2A} receptor response. The structural features required for activity were found to be highly selective. The presence, position, and stereochemistry of the Δ^9 *cis* double bond are required and even subtle structural variations reduce or eliminate activity, Fig. (**13**). Secondary or tertiary amides may replace the primary amide but follow a well defined relationship requiring small amide substituents suggesting that the carboxamide serves as a hydrogen bond acceptor but not donor. Alternative modifications at the carboxamide including acids, esters, aldehydes, alcohols, amines, acetals, or electrophilic ketones as well as modifications of the methyl terminus or the hydrocarbon region spanning the carboxamide and double bond typically eliminate activity. A less extensive study of the 5-HT_{1A} potentiation revealed that it is more tolerant and accommodates a wider range of structural modifications [42]. An interesting set of analogs was identified that inhibit rather than potentiate the 5-HT_{2A}, but not the 5-HT_{1A}, receptor response further suggesting that such analogs may permit the selective modulation of serotonin receptor subtypes and even have opposing effects on the different subtypes [42]. A number of FAAH inhibitors were also found to potentiate the 5-HT_{2A} but not 5-HT_{1A} receptor response [42]. In the former case, they possess dual activities that might provide especially useful therapeutics. Similarly, a subset of oleamide analogs bearing modifications at the α-carbon that are more resistant to FAAH hydrolysis were ineffective at the 5-HT_{2A} receptor, but well tolerated at the 5-HT_{1A} receptor [42]. Anandamide as well as oleyl ethanolamide were ineffective at the 5-HT_{2A} receptor, but provided 5-HT_{1A} potentiation comparable to oleamide [42]. In an additional interesting contrast, anandamide inhibits the ion-gated 5-HT_3 receptor response [72] whereas oleamide had no effect [41].

Similarly, oleamide has been reported to potentiate phosphoinositide hydrolysis in rat pituitary P11 cells expressing the 5-HT_2 receptor but to inhibit 5-HT_7 receptor-mediated stimulation of cAMP levels in HeLa cells transfected with the receptor [43]. In these efforts, oleamide was shown to act as a weak agonist at the 5-HT_7 receptor but to



- Increases receptor efficacy
  5-HT$_{1A}$, 5-HT$_{2A}$, 5-HT$_{2C}$ (not 5-HT$_{3}$)
  Inhibits 5-HT$_{7}$
- Does not alter serotonin ED$_{50}$
- Structurally specfic for oleamide
- Set of agents (endogenous?) which inhibit rather than potentiate some receptor subtypes
- Set which potentiate 5-HT$_{1A}$, inhibit 5-HT$_{2A}$
- Dual activity of some FAAH inhibitors

Fig. (12). Serotonin receptor modulation.

behave as an unsurmountable antagonist in the presence of serotonin illustrating that it may act at an allosteric site. Thus, oleamide has been shown to enhance (5-HT$_{1A}$ (370-550%), 5-HT$_{2A}$ (165-228%), 5-HT$_{2C}$ (365-600%)), disrupt (5-HT$_{7}$ (-50%)), or have no effect (5-HT$_{3}$) on serotonergic signal transduction at various receptor subtypes.

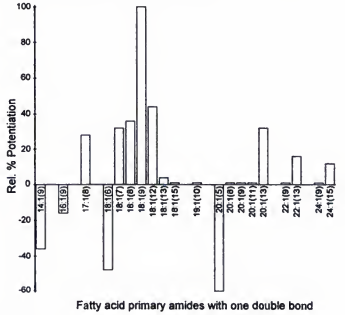
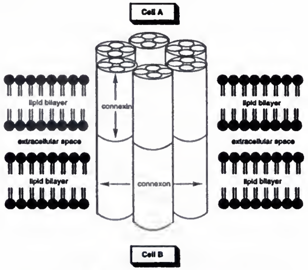


Fig. (13). 5-HT$_{2A}$ potentiation: olefin position and chain length.

Gap Junction Inhibition

Communication between cells is mediated by clusters of intercellular channels called gap junctions [73]. Gap junctions are composed of six integral membrane proteins, or connexins, that are each comprised of four α-helical bundles. Each cell synthesizes its own connexon, or hemichannel, that can interact with another connexon in the intercellular space to form a complete channel that allows cells to exchange ions, second messengers, and small metabolites. In addition to spanning two lipid membrane bilayers, gap junction channels differ from other ion channels in that they connect cytosol to cytosol instead of cytosol to extracellular space. Oleamide has been shown to inhibit gap junction communication in rat glial cells [44- 46]. Moreover, while it is capable of blocking the transfer of small molecules such as Lucifer Yellow dye and electrical transmission in rat glial cells, it does not block mechanically-stimulated or glutamate-induced Ca^{+2} ion waves thereby decoupling two previously indistinguishable communication pathways, Fig. (14) [44].



- Special cell surface protein structure that permits cell-cell communication
- Passive diffusion of Mol Wt < 1000 g/mol

rat glial cell gap junction

- blocks cell-cell dye transfer: α1 and β1
- blocks junctional electrical coupling
- no effect on Ca^{+2} wave transmission
  deconvolutes previously coupled events
- α1 connexin P$_{2}$ isoform loss (phosphorylation)
- structurally selective for oleamide
  active but less potent:
    arachidonamide, anandamide, 18:1^{8}
  weakly active:
    18:1^{11}, oleyl ethanolamide
  inactive:
    18:1^{9}(trans), 18:0
    oleic acid

Fig. (14). Gap junction inhibition.

A detailed study has defined the structural features of oleamide required for inhibition of the gap junction-mediated chemical and electrical transmission in rat glial cells [45]. The effective inhibitors fall into two classes of fatty acid primary amides of which oleamide and arachidonamide are the prototypical members. Of these two, oleamide constitutes the most effective and its structural requirements for inhibition of the gap junction are well defined. It requires a chain length of 16-24 carbons of which 16-18 carbons appears optimal, a polarized terminal carbonyl group capable of accepting but not necessarily donating a hydrogen bond, a Δ^9 *cis* double bond, and a hydrophobic methyl terminus, Fig. (**15**). Within these constraints, a range of modifications are possible, many of which may be expected to improve *in vivo* properties. For example, modifications at the α-carbon which render the agents more resistant to FAAH hydrolysis were found to produce effective inhibitors [45]. A select set of agents has been identified that serve both as oleamide agonists and as inhibitors of FAAH which is responsible for the rapid inactivation of oleamide [45]. Oleamide was more potent than arachidonamide, and both were more effective than oleyl ethanolamide or anandamide. The latter simply represents one of many secondary amides that may inhibit the rat glial gap junction, albeit less effectively than oleamide, and it was found that there was no special significance to the ethanolamide [45]. In fact, the N-propyl amides in which a methyl group is substituted for the hydroxyl group were more effective suggesting the alcohol actually diminishes the inhibitor effectiveness and similar observations with anandamide have been made with CB1 cannabinoid receptor binding [74].

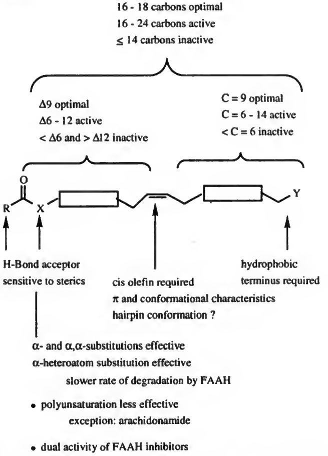
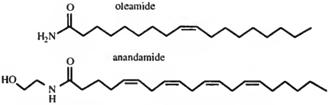


Fig. (15). Summary of structural requirements for Gap Junction inhibition.

The inhibition of the gap junction is consistent with a number of mechanisms. Perturbations in the bulk membrane fluidity or the membrane-protein interface that would affect the conformation of the membrane bound proteins as well as direct interaction with the gap junction have been discussed [75]. Such membrane or membrane-protein interface effects could also be responsible for the serotonin receptor modulation. Similarly, both progressive closure and ultimate collapse of the gap junction as well as gated closure have been considered. Structural studies conducted in the presence and absence of oleamide may serve to distinguish among the possibilities [64].

Oleamide/Anandamide Characteristics: Analgesic and Cannabinoid Effects

Oleamide and anandamide cause similar pharmacological effects in mice [48]. Although the sleep inducing properties of anandamide are not known, the CB1 receptor antagonist SR141716A increases wakefulness at the expense of SWS and REM sleep and it has been suggested that an endogenous cannabimimetic may regulate the organization of the sleep-wake cycle [76]. Oleamide exhibits essentially the same activity profile as anandamide in the four *in vivo* assays [77] commonly enlisted for characterizing the activity, Fig. (**16**). However, oleamide does not bind to the CB1 or CB2 cannabinoid receptors [48,77]. Mechoulam and coworkers have suggested that oleamide exerts these effects, and its sleep-inducing properties, through inhibition of FAAH catalyzed hydrolysis of endogenous anandamide [48]. While provocative, such an indirect diversional role for oleamide seems unlikely. Equally plausible is the possibility that both oleamide and anandamide exert their effects through a common mechanism albeit not exclusively through the studied cannabinoid receptors. Although provocative, the partial versus full CB agonist properties of anandamide and instances of noncorrelations between CB receptor binding affinity and *in vivo* potency in the tetrad of mouse assays may reflect activities derived from interactions with such targets not yet recognized [78,79]. Consistent with such a possibility, the CB1 receptor antagonist SR141716A has been shown to block Δ^9-tetrahydrocannabinol (THC) antinociception, but has no effect on anandamide [80].



Behavior effects of Δ^9-THC, anandamide and oleamide

Test ED$_{50}$ (mg/kg)	Δ^9-THC	anandamide	oleamide
Open field (ambulation)	7.0	3.4	5.7
Ring immobility	2.3	5.0	18.9
Hypothermia	9.5	3.6	17.5
Hot plate	10.3	28.0	29.2

- Identical effects in the tetrad of cannabinoid tests
- Anandamide binds to CB1 receptor ED_{50} = 39 nM
- Oleamide does not bind to CB1 receptor ED_{50} > 10 μM

Fig. (16). Oleamide and anandamide comparisons.

Note added in proof: Recently, oleamide has been shown to potentiate the response of benzodiazepine-sensitive $GABA_A$ receptors (α1β1γ2s, α1β2γ2s) to GABA but not that of simple GABA receptors or other ligand-gated ion channels including the NMDA receptor (NR1 + NR2A or 2C) and $5\text{-}HT_3$ receptor when expressed in Xenopus oocytes [81].

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List of Abbreviations

CNS = Central nervous system
FAAH = Fatty acid amide hydrolase
CSF = Cerebrospinal fluid
EEG = Electroencephalogram
DSIP = Delta-sleep-inducing peptide
SWS = Slow wave sleep
REM = Rapid eye movement
HPLC = High performance liquid chromatography
FABHRMS = Fast atom bombardment high resolution mass spectrometry
serotonin, 5-HT = 5-Hydroxytryptamine
PMSF = Phenylmethylsulfonyl fluoride
EDTA = Ethylenediamine tetracetic acid

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The Inhibitory Glycine Receptor: Prospects for a Therapeutic Orphan?

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Abstract: The inhibitory glycine receptor is a member of the ligand-gated ion channel superfamily. It mediates inhibitory synaptic transmission in mammalian spinal cord and brainstem. Structure and function of the receptor, as well as its chromosomal localization and genetic structure, have been extensively studied. While hereditary and acquired receptor dysfunctions can be identified, selective and specific modulation of receptor function is still lacking. The preponderance of current literature regarding the inhibitory glycine receptor raises the prospect that adequate methods for the treatment of glycine receptor-mediated disorders might be developed.

Introduction

The inhibitory glycine receptor (GlyR) mediates fast synaptic signal transmission in mammalian spinal cord, brain stem and other CNS regions. Receptor topology and amino acid composition of GlyR subunits classify this receptor as a member of the ligand-gated ion channel superfamily. It is intrinsically coupled to an anion channel which allows mainly chloride ions to permeate, thereby inducing postsynaptic hyperpolarization.

Localization and characterization of the glycine receptor as well as demonstration of its involvement in CNS inhibitory signal transmission were greatly facilitated by the availability of the selective high-affinity ligand strychnine. Strychnine potently inhibits the glycine receptor; in fact, the symptoms of hereditary glycine receptor dysfunction resemble strychnine intoxication.

It should be noted at this point that the NMDA receptor, a ligand-gated ion channel, belonging to the glutamate receptor family, possesses a high-affinity glycine binding site (K_D 100 nM), and requires glycine as co-agonist. This receptor site is sometimes referred to as "glycine receptor", or "non-strychnine-sensitive glycine receptor" in the literature. However, it is not subject of this review.

Understanding of glycine receptor function, its synaptic organization and developmental regulation has been greatly advanced in recent years. The recent discovery that hyperekplexia and related motor disorders are caused by defects in glycine receptor genes has opened stimulating new research opportunities.

The Support Staff of Glycinergic Neurotransmission: Transmitter Synthesis, Storage, Presynaptic Release, and Reuptake

The neurotransmitter pool of glycine is derived from both metabolic precursors and reuptake from the synaptic cleft. In the CNS, synthesis of glycine from serine is catalyzed by the mitochondrial isozyme of serine hydroxymethyltransferase [1] which, in humans, is encoded by a gene localized to chromosomal region 12q13 [2].

Glycine is predominantly degraded by the glycine cleavage system, an enzyme complex found intra- and extramitochondrially [2]. Mutations of its genes may result in non-ketotic hyperglycinemia, a devastating neonatal disease characterized by lethargy, seizures and mental retardation in surviving patients [3].

Glycine is rapidly taken up from the synaptic cleft into the presynaptic terminal by sodium-dependent high-affinity transporter systems which constitute the primary means of inactivating synaptic glycine. The transporters GLYT1a and GLYT1b, which represent splice variants of the same gene, show a widespread distribution throughout the CNS [4]. In the adult rat CNS, the transporter GLYT2 was found to colocalize with glycine receptors [5].

The onset of expression of GLYT1 and GLYT2 is in late fetal life; adult levels of transporter mRNA are reached at the age of one month [6]. Both transporters are expressed in spinal cord, brainstem and cerebellum, while moderate expression of GLYT1 is also found in cortex and hippocampus [6]. The apparent parallelism of the distribution of specific isoforms of both, receptors and transporters for glycine hints at distinct, tissue-specific roles for both transporters.

The release of glycine from presynaptic terminals is highly regulated. Glycine is stored in small synaptic vesicles [7], and released into the synaptic cleft, as shown by inhibitory miniature potentials recorded from rat motoneurons [8]. The presynaptic vesicle protein synaptobrevin is pivotal for vesicle fusion during exocytosis. Tetanus, a disease caused by the anaerobic, spore-forming rod *Clostridium tetani*, is associated with a presynaptic block of glycine release. The clostridial protein, tetanus toxin, possesses a Zn^{2+}-protease activity which selectively cleaves synaptobrevin and thus interferes with presynaptic secretion [9].

Glycine Receptors: Protein Variants and Subunit Genes

Fast excitatory and inhibitory signal transmission in the central and peripheral nervous system is mediated through

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ligand-gated ion channels. In the CNS of adult mammals, two locally and functionally well-defined inhibitory receptor types are known: Glycine receptors prevail in spinal cord and brain stem [10-12], where they are chiefly involved in regulation of motor functions. Additionally, glycine receptors are found in sensory pathways of the spinal cord as well as the auditory and visual system. Inhibitory signals of higher brain functions are generally transmitted through γ-aminobutyric acid (GABA) receptor channels; GABAergic synapses are abundant in cortex and cerebellum.

Glycine exerts its inhibitory function by binding to postsynaptic neurotransmitter receptors associated with an intrinsic anion channel. It should be noted that the physiological effect of these receptor ion channels is determined by the equilibrium potential for chloride (pCl^-). In CNS neurons of adult animals, pCl^- drives an inward chloride flux. Upon receptor channel activation, the resulting chloride current hyperpolarizes the membrane potential and depresses neuronal firing [13]. Glycine-mediated inhibition is efficiently antagonized by the convulsant alcaloid strychnine, a high affinity ligand of postsynaptic glycine receptors [10-12, 14].

During early CNS development, cortical neurons have high intracellular chloride concentrations [15], resulting in a reversed pCl^-. Consequently, glycine receptors then have an excitatory function [16] and may indeed play a role in neocortical development [17].

Receptor Structure and Isoforms

Two major isoforms of the inhibitory glycine receptor exist in rodent spinal cord. The neonatal glycine receptor is predominantly expressed around birth, and is replaced by the adult receptor form within two weeks after birth. Of these isoforms, only the adult receptor is highly to strychnine [18].

The adult glycine receptor isoform ($GlyR_A$) from spinal cord of adult mammals is a complex glycoprotein of >250 K molecular weight [19-21] comprizing three polypeptides with masses of 48 kD, 58 kD, and 93 kD [19, 22]. The ligand binding 48 kD ($\alpha 1$) subunit and the 58 K (β) polypeptide are integral membrane proteins which assemble into the pentameric chloride channel structure [23] of the adult glycine receptor variant from adult spinal cord. Radioligand binding studies show that glycine and strychnine incorporate into the 48 kD ($\alpha 1$) subunit of the receptor [19, 20, 22, 24, 25]. Upon reconstitution in phospholipid vesicles, the isolated receptor mediates glycine-activated anion translocation which can be blocked by strychnine [26]. Gephyrin, the 93 kD protein co-purifying with the glycine receptor is a tubulin-binding protein which connects the receptor to the cell skeleton. It has been implicated in spatial organization and anchoring of inhibitory synapses [27-35].

In spinal cord of newborn rodents, a GlyR variant prevails, which represents a homopentamer made up of polypeptides ($\alpha 2$) with a molecular weight of 49 kD [36]. This neonatal receptor isoform ($GlyR_N$), which is characterized by low strychnine binding affinity, accounts for about 70 % of total glycine receptor in spinal cord of the newborn rat, and is fully replaced by the adult receptor within two weeks after birth [36] (see also [37, 38]). It could be shown through biochemical [21, 36] and electrophysiological [39] studies that rodent and human $\alpha 2$ receptor subunits are highly homologous.

Developmental changes in strychnine sensitivity of rodents are thought to reflect alterations in glycine receptor isoform expression [36, 39]. Likewise, primary cultures of spinal cord predominantly express the neonatal isoform of the glycine receptor protein [21], and strychnine resistant, glycine-activated currents have been recorded from cultured neurons [40]. Developmental changes of glycine receptor isoform expression overlay with alterations in pCl^- [17].

In addition to developmental changes observed for glycine receptors, genetic differences within one species may also contribute to receptor complexity. Glycine receptors from two strains of mice, long sleep and short sleep (LS and SS) were found to have different sensitivities to ethanol and anaesthetics [41].

Glycine Receptor Subunit Genes

GlyR protein complexes assemble from a variety of ligand binding ($\alpha 1$-$\alpha 4$) and structural (β) subunits (see table 1). With respect to primary structure and transmembrane topology, these subunits are highly homologous to each other [39, 42-46], and to other ligand-gated ion channels, such as $GABA_A$, nicotinic acetylcholine, and $5\text{-}HT_3$ receptors. Homologies between subunits from different ligand-gated ion channel receptors are in the range of 30 to 70 % [47]. These observations indicate that the ligand-gated neurotransmitter receptor superfamily evolved from common ancestral genes [42, 47-50].

Chromosomal localization and architecture of the human and murine glycine receptor genes have been identified (see table 1). All α subunits share a 9-exon structure of their open reading frame. Receptor complexity is further increased by alternative splice sites between exon 8 and 9, which were demonstrated for $\alpha 1$ [51] and $\alpha 3$ [52] subunits. This gene region codes for amino acids in the cytosolic loop between transmembrane segments TM 3 and TM4. Alternative splicing removes a segment of 15 amino acids containing a phosphorylation consensus sequence [52]. Similar splice variants have been observed for the $5\text{-}HT_3$ receptor [53].

Table 1. Glycine Receptor Subunit Genes

Subunit	Chromosomal locus		Reference
	Human	Mouse	
$\alpha 1$	5q31.2	11(29 cM)	[71]
$\alpha 2$	Xp21.2-p21.3	X (70 cM)	[71]
$\alpha 3$	4q33-q34	8 (25 cM)	[52] / [71]
$\alpha 4$	n.d. (Xq21-22?)	X (56 cM)	[71]
b	4q31.3	3 (36 cM)	[274] / [71]

Note: lit. [274] is:
Milani, N.; Mulhardt, C.; Weber, R. G.; Lichter, P.; Kioschis, P.; Poustka, A.; Becker, C.-M. *Genomics*, **1998**, *50*, in press.

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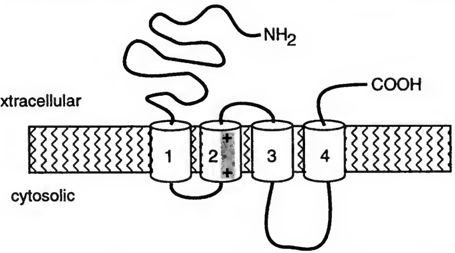


Fig. (1). Glycine receptor transmembrane topology. Charged amino acids and channel-lining segment of transmembrane domain TM2 are indicated.

Receptor Distribution and Developmental Regulation

Probing GlyR Distribution and Expression

Glycine-displaceable [^{3}H]strychnine binding is a reliable probe of the inhibitory glycine receptor. Since strychnine is a highly selective glycine receptor ligand, high-affinity strychnine binding found in a tissue sample is generally assumed to indicate the presence of glycine receptors. Moreover, the availability of specific antibodies and hybridization probes has permitted detailed analyses of glycine receptor distribution. Glycine application affects many processes in the developing and mature nervous system, and frequently such effects are interpreted as glycine-receptor mediated. Thus, the reports of strychnine binding or functional effects of glycine discussed in this chapter certainly present strong evidence for glycine receptor involvement, even if this may not in every case constitute final proof.

GlyR in Spinal Cord and Brain Stem

In synaptosomal membranes from spinal cord and other CNS regions, [^{3}H]strychnine recognizes a single class of binding sites with affinity constants of 3 - 10 nM. Strychnine effects on motoneuron activity in rat spinal cord were monitored using calcium imaging [54]. Rhythmic motor activity in spinal cord was affected by strychnine application [55]. Contralaterally elicited postsynaptic potentials in developing rat brainstem were blocked by strychnine, suggesting an involvement of glycine receptors in this tissue [56]; similarly, spontaneous motoneuron activity in fetal rat spinal cord was strychnine-sensitive [57]. Glycine application changed firing patterns in specific rat spinal cord neurons [58]; likewise, hindlimb locomotor patterns in the neonatal rat were mediated by strychnine-sensitive glycine receptors [59]. GABA and glycine release in rat spinal cord was suggested to be involved in the regulation of both background neuronal activity and response to mechanical stimulation, since the corresponding neuronal activities were affected by strychnine [60], or by both strychnine and the GABA-receptor antagonist bicuculline [61].

As pointed out before, native glycine receptors comprise two major protein isoforms which underly developmental regulation: The neonatal isoform (GlyR$_N$) is fully replaced by the adult receptor form (GlyR$_A$) within two weeks after birth [36]. The subunit compositions of both receptor isoforms have been identified [21, 36, 62]. GlyR$_N$ appears to be a homopentamer consisting of $\alpha2$ subunits, while the adult receptor isoform is a complex heteropentameric glycoprotein of $\alpha1$ and β subunits. The neonatal receptor protein variety displays a low affinity for strychnine, in contrast to the adult rodent spinal cord glycine receptor. Such a developmental regulation of glycine receptor isoforms has also been found in neurons of rat cerebral cortex [63, 64].

Developmental changes in strychnine sensitivity of rodents have been attributed to alterations in glycine receptor isoform expression [36, 39]. Using non-radioactive hybridization, glycine receptor isoform distribution was studied in the cochlear nucleus and the superior olivary complex of 5-6 week old and 8-10 week old rats. $\alpha1$, $\alpha3$ and β subunits were found in all major neurons from 8-10 week old rats, while after 6 weeks, the neonatal $\alpha2$ receptor form was no longer expressed [65]. In the cochlear nuclei of Fisher rats, on the other hand, the *total* number of receptors was downregulated with age, as determined by strychnine binding: between 3 months and 26 months of age, the number of strychnine binding sites was reduced by 30 % [66]. A developmental replacement of $\alpha2$ subunits by $\alpha1$ was found when the expression of glycine receptor mRNA in rat spinal cords was studied by *in-situ* hybridization [67].

Firing patterns in the respiratory network of frogs [68] and rats [69] are affected by strychnine. In mature mice, topical strychnine application to the ventrolateral medulla altered the breathing pattern; interestingly, in neonatal mice (up to P15), strychnine was without effect [70], a finding that agrees with the observed pattern of glycine receptor isoforms expression in mice.

The expression pattern of glycine receptor isoforms in the rodent CNS is developmentally regulated at the transcriptional level [71]. Akagi et al. isolated two classes of poly(A)$^+$-mRNA from brain and spinal cord of fetal and adult rats. Fractionation of mRNA and expression in *Xenopus laevis* oocytes resulted in two different glycine-gated chloride channels, corresponding to the neonatal and adult receptor type [72-75]. Developmental analysis of defined glycine receptor subunit transcripts reveals that spinal cord levels of both α1 and α3 subunit mRNAs increase postnatally [44]. At all times, mRNA expression of the a1 subunit exceeds levels of α3 transcripts [76]. In contrast, α2 transcripts are abundant around birth and display a drastic postnatal decrease [39]. Results from RNA studies are supported by functional analysis of the corresponding recombinant proteins as well as the native receptors.

Cultured Spinal Cord Neurons

Primary cultures of embryonic spinal cord neurons predominantly express the neonatal isoform $GlyR_N$ of the glycine receptor protein [21]. Strychnine resistant, glycine-activated currents have been recorded from cultured neurons [40]. The developmental pattern of GlyR isoform expression in rodents could be reproduced in *in vitro* cultures of embryonic rat spinal cord neurons. Whole-cell recording and immunodetection with the monoclonal antibody mAb4a, which binds to rat α subunits, revealed the presence of glycine receptors already on day 1 in culture. Strychnine sensitivity of glycine-evoked whole-cell currents, however, was only observed after 8 days in culture, confirming the existence of a strychnine-insensitive embryonic receptor form which is replaced by the adult receptor during development [77]. Differential expression of glycine receptor subunits during differentiation of mouse P19 embryocarcinoma cells, as studied by RT-PCR and immunocytochemistry, showed that the β subunit is constitutively expressed, and α1 and α2 are induced, while α3 could not be detected [78]. The distribution of mRNA for glycine receptor subtypes *within* neurons of the ventral horn of the rat spinal cord was studied using electron microscope *in situ* hybridization (ISH). α subunit mRNA was found in both somata and dendrites while mRNA for the β subunit and gephyrin was only found in the somata of these neurons [79]. Glycinergic transmission regulates the growth of postsynaptic processes in an organotypic culture of the medial nucleus of the trapezoid body and the lateral superior olive of gerbils. Addition of strychnine to the culture medium led to dramatically increased dendritic branching and dendritic length [80]

Cortex

Two classes of $[^3H]$strychnine binding sites were reported for rabbit cortex [81]. Glycine receptors were identified in cultured postnatal hippocampal neurons by electrophysiological methods [82]. Glycine receptors in the developing rat neocortex were shown to have an excitatory function, due to higher intracellular chloride concentrations in these tissues [15, 16]. These receptors seem not to be involved in a functional transmitter system. However, non-synaptically released taurine has been strongly suggested as endogenous ligand for these receptors. Indeed, glycine receptors may be part of a novel signalling system in early cortical development [17].

Retina

Glycine and strychine binding studies suggest the existence of glycine receptors in synaptosomal membranes from frog retina [83]. In rat retina, mRNA of the glycine receptor subunits α1, α2, α3, β, and for gephyrin could be detected, while in rod bipolar cells only mRNA for α1 and β subunits (not for gephyrin) was found [84]. In retinal ganglion cells, two discrete glycinergic signalling pathways were identified, one of which was sensitive to strychnine [85]. Glycine receptors were identified in monkey retina using the α1 subunit-specific antibody mAb2a [86], and in retina of developing chickens [87]. Glycine receptor localization in the retina of albino rats was studied using antibodies specific for α1 (mAb2a), as well as for all glycine receptor α subunits (mAb4a). The observed punctate imunofluorescence could be shown to represent glycine receptor clustering at synaptic sites [88].

Auditory System

Strychnine application affected the function of the echo-delay system in mustached bats [89]. Developmental regulation in the rat cochlear nucleus and superior olivary complex of glycine receptor isoforms [65] and the total number of receptors [66] has already been discussed.

Glia and Extraneural Effects

In glial cells from rat spinal cord, glycine receptor subunits α1 and β could be identified using patch-clamp techniques in combination with single cell RT-PCR, demonstrating the presence of functional receptors in glia. In culture, however, glial receptor expression is lost indicating that it requires stimuli from the natural spinal cord environment [90].

Apart from their widespread distribution throughout the CNS, glycine receptors were recently identified *outside* the nervous system: Glycine-gated chloride channels were identified in porcine and human sperm, where they are suggested to be involved in the acrosome reaction [91-93], and in Kupffer cells of the liver [94].

The Function of Gephyrin: Organizing the Glycinergic Synapse?

During purification of the native glycine receptor by affinity chromatography, gephyrin, a 93 kD protein which is strongly associated with the receptor, was co-purified with the membrane-spanning α and β subunits [19, 22, 27]. Gephyrin was localized to the cytoplasmic side of the postsynaptic membrane [95, 96]. As a peripheral, tubulin-binding membrane protein, it reversibly associates with the transmembrane core of the receptor and has been implicated in its synaptic anchoring [27, 28]. Using photobleach recovery of fluorescent strychnine derivatives, a laterally mobile and an immobile glycine receptor population could be distinguished on cultured neurons [97]. It is tempting to speculate, although not proven, that association with gephyrin links the receptor to the cytoskeleton, thereby fixing its position. A model has been proposed which requires glycine receptor activation, resulting in Ca^{2+} influx, for the clustering of gephyrin and glycine receptors at the developing glycinergic synapse [35].

A gephyrin-binding motif consisting of an 18 amino acid stretch in the cytoplasmic loop between transmembrane segments 3 and 4 was identified on the glycine receptor β subunit [98]. Neither glycine receptor α subunits nor $GABA_A$

receptor β1 subunits showed gephyrin binding in this study. Gephyrin could be detected by immunostaining in the retina, but not in rod bipolar cells of rats [84]. Often it is found in the same cells as glycine receptor subunits, although not necessarily localized at the same position. In general, expression of gephyrin and glycine receptors coincides, and a developmental regulation for gephyrin was observed [99, 100]. From these observations, gephyrin was proposed as a specific marker for glycine receptors and glycinergic synapses.

However, gephyrin was found to colocalize with glycinergic *and* GABAergic synapses in cat spinomedullary neurons [101], rat retina [102, 103], and rat spinal cord [104]. Furthermore, in cultured rat hippocampal neurons, where glycinergic signal transmission is absent, gephyrin was found to colocalize with GABAergic, but not glutamatergic synapses [105].

It would thus be tempting to infer that gephyrin is at least a selective marker of inhibitory synaptic contacts. However, a recent immunostaining study found agrin (a protein involved in the aggregation of nicotinic acetylcholine receptors) adjacent to gephyrin [106]. A widespread distribution of gephyrin in most mammalian tissues has been inferred from an analysis of the gephyrin gene promoter region [107].

In addition to their task as synaptic organizers, gephyrin and other synaptic proteins might play a much more general role within the organism than was generally assumed.

Glycine Receptor Disorders: Pathological Windows to Receptor Function

Glycine Receptor Mutations in the Mouse

Identification of mutations in murine glycine receptor subunit genes have allowed a comprehensive correlation of gene function and phenotype [18]. In the *spastic (spa)* mouse, intronic insertion of a LINE-1 element into the β subunit gene causes aberrant splicing of transcripts resulting in a reduction of *Glrb* the number of functional glycine receptors. The *spasmodic (spd)* mouse carries a missense mutation in the α1 gene *Glra1*, which gives rise to normal expression levels of receptors with reduced agonist affinity. In the *oscillator* (*spd^{ot}*) mutant, a microdeletion within the α1 gene *Glra1*, resulting in a translational frameshift, causes complete loss of GlyR_A; homozygosity for this mutation results in lethality. Phenotypes of all these mutants are characterized by hypertonia, myoclonia, and excessive startle responses [18], particularly to acoustic stimuli [108]. The symptoms only become manifest after 2 weeks of age, indicating that the neonatal, less strychnine-sensitive form of the glycine receptor is not affected. It may be safe to assume that a defect in the gene for the embryonic glycine receptor is lethal in the early stages of fetal life.

Hyperekplexia Mutations of the GLRA1 Gene: Clues to Glycine Receptor Function and Regulation in the Human

Hyperekplexia (startle disease, stiff baby syndrome) is a congenital motor disorder resembling the murine phenotypes [18, 109, 110]. Dominant and recessive forms of this disease result from missense mutations of the *GLRA1* gene (Chr 5) that affect affinity and/or ion conductance of the encoded receptor channel [111-116]. In a recessive case of hyperekplexia, a null allele of *GLRA1*, was identified, consistent with a complete loss of gene function. Despite this *"knockout"* situation, the affected child displays relatively mild symptoms [117]. In contrast to the lethal effect of a *null* allele in the *oscillator* mouse, the complete absence of GlyR α1 subunits is tolerated in the human suggesting that either the loss of GlyRs is effectively compensated or that subunit regulation substantially differs among these species.

Metabolic Disorders

Glycine receptor density, usually measured by [^{3}H]strychnine binding, is affected in several metabolic and neurodegenerative diseases [18]. Receptor densities increase in murine hyperglycinemia [37]. Neurodegeneration is thought to cause loss of glycine receptors in the substantia nigra of Parkinson patients [118], and in the spinal cord of persons suffering from amyotrophic lateral sklerosis [119]. Peripheral axotomy also leads to loss of glycine receptors which, however, reappear after nerve regeneration [120]. The autoantigen NOVA-1, a RNA-binding protein which is involved in paraneoplastic opsoclonus myoclonus ataxia (POMA) was found to bind to glycine receptor α2 pre-mRNA with high specifity, suggesting that this subunit may be involved in the motor dysfunction seen in the disease [121].

The Glycine Receptor Under a Molecular Microscope: Functional Domains

Ligand Binding

Recombinant expression of glycine receptor α subunit variants leads to the formation of homooligomeric ion channels whose pharmacological profile is indistinguishable from native receptors. Strychnine sensitivity, as well as dose-response relationships for glycine, β-alanine, and taurine are conserved [39, 44, 46, 122, 123], although single-channel conductances of homomeric (α1) and heteromeric (α1 plus β) recombinant glycine receptors expressed in HEK 293 cells exhibit different single-channel conductances.

Photoincorporation of [^{3}H]strychnine into membrane fractions from adult rat spinal cord and subsequent fractionation showed that strychnine only bound to the α1 subunit [19, 20, 24, 25] . This covalent incorporation is specifically antagonized by glycine [25]. Proteolysis of [^{3}H]strychnine-photolabelled synaptosomes removed the first ca. 100 amino acids of the extracellular domain of the α1 subunit [25], leaving the radiolabel still attached. This part of the polypeptide was, therefore, considered not to contain the ligand-binding domain of the receptor [42]. Ligand binding to the glycine receptor α subunit is tentatively assumed to occur in a region of the N-terminal domain immediately preceding the first transmembrane segment, M1 [42, 48].

The fact that homomers of an α2 glycine receptors from rodents exhibit a low affinity for strychnine, while the highly homologous human α2 receptor is sensitive to convulsants has led to the identification of a crucial determinant of receptor ligand-binding: the exchange of a single amino acid in position 167 of the human α2 glycine receptor from glycine to glutamate renders this subunit strychnine-insensitive. Reversing this exchange by site-directed mutagenesis restores strychnine sensitivity to the murine α2 (E167G) variant [39]. Position 167, therefore, although distant from the strychnine-incorporation site, appears to crucial for high-affinity strychnine binding [48]. The strychnine-insensitive α2 subunit, variant, which carries a glutamate in the corresponding position, prevails in rodent spinal cord.

In the α subunit of the nicotinic acetylcholine receptor, acetylcholine binding depends on a stretch of amino acids, including two neighbouring cysteines, that precedes the first transmembrane segment. A sequence comparison shows that this region of the acetylcholine receptor corresponds to the pre-M1 domain of the highly homologous glycine receptor [48]. Two cysteines, which are conserved and exactly 13 amino acid residues apart in all known α subunit variants of glycine, $GABA_A$, and nicotinic acetylcholine receptors are thought to form a disulfide bridge essential for receptor conformation [48]. In the case of the glycine receptor, tyrosine residues were shown to be essential for strychnine binding [124]. These structural elements, together with several charged amino acid residues provide functional determinants of ligand recognition [42, 48]. Attempts have been made to model the arrangement of hydrophobic, aromatic and electrostatic interactions for receptor-ligand binding and condense these into a binding site pharmacophore [125, 126]. Since all required structural elements are present in the N-terminal extracellular domain of α1, this domain has been proposed by Grenningloh et al. [42] to be the site of strychnine binding. Analysis of proteolytic fragments of the glycine receptor has indeed proven that UV-illumination leads to incorporation of $[^3H]$strychnine into peptide fragments from this subunit region [127].

Chemical protein modifications of the receptor affect binding of glycine and strychnine differently. Treatment with diazonium tetrazole (modification of histidyl and tryptophanyl residues) or the amino-reactive agent acetic anhydride deletes displacement of $[^3H]$strychnine binding by glycine but not by unlabelled strychnine in rat spinal cord membranes [124, 128]. Modification of lysine by fluorescein 5′-isothiocyanate (FITC) also affects glycine- but not strychnine-binding, indicating that agonist and antagonist are recognized by distinct amino acid residues [129]. Attempts to map the incorporation site of fluorescent label within the α subunit sequence were, however, not successful [129]. Glycine and strychnine binding was investigated in mouse spinal cord membranes, and the protein modifying agents N-bromosuccinimide, tetranitromethane, diethylpyrocarbonate and 2,3 butanedione were used under conditions selective for tryptophan, tyrosine, histidine, and arginine residues, respectively [130]. Ligand-binding to the receptor as well as strychnine binding to strychnine-specific antibodies was reduced after protein modification. The results obtained suggest that glycine and strychnine bind to distinct but overlapping sites on the receptor with tyrosine, tryptophan, histidine and arginine residues as strychnine contact residues, while histidine is also implicated in glycine recognition [130]. Analysis of ion effects on the glycine receptor further supports the concept of non-identical agonist and antagonist binding determinants since, for instance, sodium chloride increases strychnine but lowers glycine binding affinity. Taken together, biochemical and molecular cloning studies have permitted a regional localization of α subunit domains involved in ligand binding whereas determinants of agonist recognition are not defined.

In the case of the nicotinic acetylcholine receptor, the ligand-binding region has been located to the interface region between adjacent subunits (see [47] for review). Residues at the α-δ and α-γ subunit interfaces were identified by photoaffinity labelling studies [131] crosslinking and site-directed mutagenesis experiments [132-134] to be critical for ligand binding. Data for the $GABA_A$ receptor are less conclusive, but a similar overall structure of the binding site has been inferred from mutagenesis studies and sequence homology between these receptors, particularly since a minimal expression of α *and* β subunits is thought to be required for reconstruction of native gating properties in recombinant $GABA_A$ receptors [47]. In the case of $5\text{-}HT_3$ [135] and neuronal nicotinic acetylcholine α7 [136] receptors, however, recombinant expression of a single subunit results in functional ion channels with properties similar to native receptors. In the case of the inhibitory glycine receptor, however, all structural information for ligand binding resides in a single (α) subunit. Nevertheless, ligand binding at the interface between two subunits may still be the basis for ligand recognition of both homomeric and heteromeric receptors.

Thermodynamic parameters of ligand-receptor interaction have been identified which are assumed to reflect corresponding energy changes of the receptor complex. Higher temperature increases receptor affinites for glycine, β-alanine, and taurine i.e. formation of the receptor-agonist complex is favoured under these conditions. In contrast, affinities for strychnine and other antagonists are significantly reduced at elevated temperatures. Further thermodynamic analyses indicate that glycine receptor agonist binding is driven by increases, and antagonist binding by decreases in enthalpy (H°) [137]. Thermodynamic parameters might, therefore, be useful for the characterization of agonist and antagonist binding, and may help predict how compounds may interact with the inhibitory glycine receptor.

The Glycine Receptor Ion Channel

Ion Pore

Hydropathy profiles of the mature glycine receptor protein (all subunits) predict four hydrophobic segments (M1 to M4) spanning the postsynaptic membrane [42]. Within the predicted transmembrane regions of glycine receptor subunits, segment M2 is considered to be the pore-lining polypeptide chain of ligand-gated ion channel proteins [11, 47-49]. A ring of positively charged amino acids at the presumptive mouth of the channel has been suggested to be the structural correlate of anion binding sites [11] within the channel that were previously postulated from electrophysiological studies [138].

Measuring the permability of large, multiatomic ions, the pore dimensions of ion channels can be estimated [139-141]. Using this approach, the pore size of the glycine receptor channel in mouse cultured spinal neurons was found to be 5.2 Å, nearly equal to that of the inhibitory $GABA_A$ receptor pore [138]. Mutation of amino acids within the M2 segment altered the conductance of homomeric glycine α1 receptors in HEK 293 cells [142]. A synthetic peptide, C-K4-M2GlyR, which mimics the M2 segment of the glycine receptor α subunit with four additional lysine residues added to the carboxy terminus for increased solubility was synthesized. Upon incubation with a monolayer of Madin-Darby canine kidney cells, transmembrane fluxes of chloride and fluid were observed, which could be blocked by Cl^- channel inhibitors [143].

Conductance States

Conductance states of 29, 18, and 10 pS were found for glycine receptors from cultured mouse spinal cord neurons [138, 144]. Another study found a main conductance state of 42 pS, and a subconductance state of 27 pS for the receptor, both of which had a similar gating mechanism [145]. Kinetic studies found double-exponential receptor desensitization [146, 147] and recovery from desensitization [146]. Two independent phases of glycine-induced whole-cell currents were identified on embryonic mouse spinal cord cells, only one of which was sensitive to picrotoxin. Agonist dissociation constants of 220 µM and 380 µM, and maximum desensitization rates of 0.7 s^{-1} and 0.1 s^{-1}, respectively, were found for the two current components [147]. These observations, obtained by functional techniques, showed the presence of multiple, independent receptor subtypes in embryonic murine spinal cord [147].

Channel Gating

Ligand-gated ion channels open and close on a micro- to milli-second time scale. Therefore, thermodynamic parameters alone may not suffice to describe receptor-ligand interactions adequately. Since equilibrium methods only allow study of a mixed population of active and desensitized receptor states, rapid kinetic techniques were introduced to study the elementary steps of receptor function with appropriate time resolution [148]. Caged derivatives of glycine and β-alanin were synthesized [149-151], which should eventually allow determination of the individual rates of glycine receptor channel opening and closing.

The vast majority of studies of glycine receptor function make use of electrophysiological methods. Measurements of radioactive chloride uptake have recently been suggested as a less invasive technique that would not be restricted to immature, cultured neurons, as are, allegedly, patch-clamp techniques [152]. Measuring ^{36}Cl uptake into vesicles from spinal cord and brainstem from adult mice, two components of glycine-stimulated uptake were found with EC50 values of 22 µM and 0.5 µM respectively, which were antagonized by strychnine (IC_{50} 0.4 µM) [152]. It remains to be seen if these experiments [152] describe new receptor isoforms with drastically different ligand affinities, or if the technique used measures a distribution of receptor states different from that seen in electrophysiological experiments.

Receptor Structure

Functional studies of both native and recombinant receptors are well established. Due to inavailability of sufficient amounts of protein, structural information, other than that indirectly inferred from mutational or proteolytic analysis, is not yet available for ligand-gated ion channels. The only exception in this respect its the nicotinic acetycholine receptor which can be isolated in large quantities from the electric organ of several species of electric eel and ray. The nicotinic acetycholine receptor has, therefore been studied to resolutions of up to 7.5 Å [153, 154], and by sequence homology, the overall topology of the related receptor in the ligand-gated ion channel superfamily is assumed to be analogous. Remarkably, baculovirus-driven expression of glycine receptor α1 subunits in *spodoptera frugiperda* cells resulted in expression levels that were 4- to 30-fold higher than in eucaryotic systems [155]. Since the protein could be highly purified from the plant tissue, this approach may (literally!) constitute a "crystallization" point for a structure determination of the receptor. Recently, the first crystal structure of a K^+ channel was published [156], and it can be expected that even the elusive ion channel receptors will eventually yield to x-ray structure analysis.

Ion Effects on Receptor Function

Regarding ionic effects on the function of the glycine receptor complex, one has to consider four major groups of ions: (i) permeant ions that contribute to the charge translocated through the ion channel, (ii) ions that directly modulate the glycine receptor protein, thereby esentially acting as allosteric modulators, (iii) counter-ions that have an indirect effect by modifying solubilization or (thermodynamic) activities of compounds interacting with the receptor, and (iv) ions which themselves activate the receptor ion channel.

The general understanding of the pore structure of ligand-gated ion channels is that a ring of charged residues provides an electrostatic filter which allows only one class of ions to pass through the channel. The ease of traversing the channel depends on ion size, charge and size and stability of the hydrate shell of the ion [139].

(i) The ion pore induced by glycine binding to the pentameric receptor complex is selective for monovalent anions. In a patch clamp study on cultured neurons, a permeability sequence has been established of $SCN^- > I^- > Br^- > Cl^- > F^-$ [138]. The neurophysiological activity of these anions correlates with their channel permeability, but not with ion mobilities in free solution, which has the sequence $Br^- > I^- > Cl^- > SCN^- > F^-$. Single-channel measurements of glycine receptor channels gave a conductance sequence of $Cl^- > Br^- > I^- > SCN^- > F^-$ which is nearly the inverse of the premeability sequence lumen of channel. Further analysis showed that for pore selectivity is in fact achieved by two anion binding sites [138].

(ii) Di- and polyvalent cations were found to modulate glycine receptors [157, 158]. Zn^{2+} had a biphasic modulatory effect on glycine receptor currents in rat embryonic spinal cord neurons and recombinant receptors expressed in *Xenopus* oocytes. Low concentrations of Zn^{2+} (0.5 - 10 µM) increased glycine-evoked currents some 3-fold, while at concentrations higher than 100 µM glycine responses were decreased [157]. A minor monophasic effect of Zn^{2+} was observed in cholinergic neurons from the septal region of fetal rat brains [158]. Glycine-mediated currents in the presence of 100 µM Zn^{2+} were increased by 38 % over control values. Pb^{2+} and La^{3+} increased glycine currents 23 % and 14 % respectively [158]. These observations suggest the existence of a modulatory cation binding site on glycine receptors.

Zn^{2+} was found to affect glycinergic responses in the CNS, and has also been shown to inhibit NMDA receptors, slightly potentiate non-NMDA glutamatergic transmission, and to block certain GABA receptor channels [159]. Since Zn^{2+} is abundant in the brain (although most of it (>90 %) is bound in metal-protein complexes such as zinc finger proteins), these findings suggest a role for Zn^{2+} as an important modulator of synaptic transmission [159].

(iii) Anion binding to the glycine receptor has been postulated from functional studies. Ion permeation through the channel, was suggested to be regulated by two anion binding sites [138]. The ammonium and choline salts of several permeant monovalent anions were found to inhibit strychnine binding to the receptor with potencies of inhibition in the order formate > Br^- > Cl^- > I^- > NO_3^- > ClO_4^- > SCN^- (F^- had no effect). Hill coefficients for this inhibition of 2.3, 2.5, and 2.7 were observed for chloride, bromide and iodide, respectively, ED_{50} values were in the range from 160 to 620 mM [160]. Low ionic concentrations were not investigated in this study (see below). With the exception of formate, the neurophysiological activity of these anions correlates vaguely with their ion channel permeability [161]. Another study found biphasic effects of these anions: at concentrations below 200 mM strychnine binding was enhanced while in the concentration range of 200 mM to 3 M, strychnine binding was inhibited [162]. The biphasic anion regulation of $[^3H]$strychnine binding to the glycine receptor was interpreted in terms of a model predicting two anion binding sites [128, 162, 163].

Whenever anion effects on the receptor are studied, a pronounced effect of the counterions is observed. Inhibition of strychnine binding by ammonium salts of permeant anions is abolished by sodium [124, 163, 164]. In the presence of chloride as anion, the effect of ammonium dominates over sodium [163]. Monovalent counter-cations exhibit a biphasic modulation of strychnine affinity of the receptor: sodium, lithium and potassium salts in concentrations up to 150 mM decrease strychnine binding [164], while higher concentrations increase strychnine binding [164, 165]. Protein modification by the amino-group-selective reagent acetic anhydride eliminates the effect of sodium salts while ammonium salts remain unaffected [163], suggesting different sites of action of the two ions. The effects of ions on glycine and strychnine binding to the receptor are different. NaCl concentrations that increase strychnine affinity for the receptor reduce the binding of glycine and other agonists [165]. Protein modification by the amino-group-selective reagent acetic anhydride eliminates the effect of sodium salts while ammonium salts remain unaffected [163], suggesting different sites of action of the two ions. The effects of ions on glycine and strychnine binding. In contrast to thermodynamic parameters [137], however, the resulting shift in association constants [165] is not of predictive value for agonist or antagonist properties of glycine receptor ligands.

(iv) Surprisingly, in the presence of chloride as permeant ion Cs^+ was found to activate the glycine receptor. This activation could be blocked by strychnine [40], a finding that is not easily reconciled with the modulatory effects of ions on the receptor that is normally observed.

Effects of Receptor Environment

Apart from modulation of receptor function by ligands and ions, pressure was also found to affect the glycine receptor ion channel. Dose-response curves for homomeric $\alpha 1$ glycine receptors expressed in *Xenopus* oocytes were shifted to the right at elevated pressures, while the maximum response was not affected. The EC_{50} values in the pressure range from 0.1 MPa (atmospheric pressure) to 15 MPa (a pressure range experienced by divers) changed from 190 to 480 µM [166]. This finding is in agreement with the observation that glycine administration reduced the sensitivity to pressure-induced pain in rats, while strychnine administration led to increased neuropathic pain responses in rats [167].

The *density* of recombinant receptors expressed in *Xenopus* oocytes was found to affect agonist affinity, receptor gating and channel permeation [168].

Channel Gating and Transducer Function

The fact that even homomeric receptor channels can have multiple conductance states complicates detailed analysis of channel activity; additionally, phosphorylation can confer new gating properties on the receptor [169, 170]. The stoichiometry of receptor-ligand interaction and channel gating has to be evaluated through methods that are, to different degrees, affected by ambient parameters such as temperature, pH, ionic composition of solution, presence of modulating substances, and cytosolic and membrane receptor environment. Receptors from different animal species or tissues might have different functional properties. Cell culture of primary cells is known to affect expression of receptor isoforms [90], and recombinant expression may lead to altered function due to lack of environmental proteins such as gephyrin, or high phosphorylation background, as known for *Xenopus* oocytes [171]. Since the developmental expression pattern already shows that THE unique glycine receptor does not exist, it is not surprising that there are large numbers of conflicting results, even though data were carefully collected and analyzed.

Single amino acid mutations within the glycine receptor, observed either in hereditary receptor dysfunction or deliberately introduced into recombinant receptors, can give information about positions within the polypeptide that are crucial for ligand recognition and subsequent channel-opening of the receptor. Such experiments have to be carefully interpreted, since a single point mutation can affect the conformation and function of an entire protein. Such a situation is evident in the case of, sickle-cell anemia.

Remarkably, certain amino acid positions are critical in conferring specific properties on the glycine receptor. Mutation of an arginine residue in position α1 (271) to either glutamine or leucine (a mutation that is also found in hyperekplexia patients) not only disrupts channel activation *after* ligand-binding, but also converts the $GABA_A$ receptor antagonist picrotoxinin from a competitive antagonist to an allosteric potentiator of the glycine receptor [172]. If the same amino acid is mutated to lysine, the affinity of the receptor towards the partial agonists β-alanin and taurine was reduced. These ligands no longer activated the receptor while still competitively inhibiting receptor activation by glycine [173]. Likewise, mutation of position 52 of the α subunit (alanine to serine) alters the ethanol sensitivity of recombinant glycine receptors expressed in *Xenopus* oocytes [174]. These results support the hypothesis that the binding sites for glycine and strychnine on the receptor are overlapping but not identical. The lack of high affinity agonists, however, has to date precluded mapping of the α subunit agonist recognition site. The loop regions connecting transmembrane segments M1 with M2, and M2 with M3 are suggested to act as hinges governing control of displacement of the M2 segment, and thus controlling channel opening [175]. In fact, hyperekplexia mutations of the glycine receptor are mostly found in the region between transmembrane segments M1 and M3 [111, 175-178]. As already mentioned, modification of amino acids within the pore-lining segment M2 affects channel conductance, [176-178].

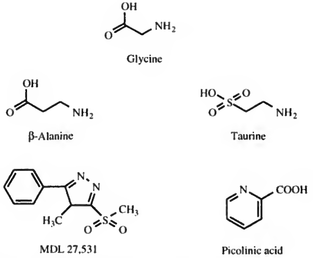
Chimeric $GABA_A$/glycine receptors were constructed, which had (i) a stretch of 30 amino acids from the N-terminus, or (ii) a 100-amino acid stretch at the C-terminus of TM3 swapped between glycine receptor α1 and $GABA_A$ receptor α2 and β subunits. The chimeric receptors were tested for GABA and glycine responses and it could be shown that neither of the two exchanged fragments is associated with barbiturate effects on $GABA_A$ receptor function [167].

Cooperativity of Glycine Receptor Function

As mentioned before, ligand binding sites may be located entirely within one subunit, or at the interface between subunits. In either case, cooperativity may affect binding of a second ligand. While no direct structural information is yet available, the accumulated biochemical evidence begins to produce consistent models of receptor-ligand interaction. Ligand-binding studies are often analyzed using the Hill equation (it should be borne in mind that the assumption made by A. V. Hill [179] is one of extreme positive cooperativity between binding of the first and subsequent ligand molecules, only then does this approximation apply). The Hill coefficient obtained from the analysis of binding, displacement, or dose-response data should, therefore, be considered to reflect cooperativity of ligand binding rather than a true estimate of the number of ligand binding sites [180].

A different approach, based on a minimum mechanism which assumes a fixed number of independent ligand-binding sites has been shown to satisfactorily describe the activation and desensitization of nicotinic acetylcholine [181-184], $GABA_A$ [185], serotonin 5-HT_3 [186] and glycine [147] receptors, and has the advantage of making the least number of assumptions regarding receptor-ligand interaction.

In case of the glycine receptor, Hill coefficients for glycine binding are usually found to differ significantly from unity, varying between 1.7 and 2.7 for glycine-mediated chloride currents recorded from spinal motoneurons [187], cultured neurons [40, 188, 189], and recombinant glycine receptors expressed in *Xenopus laevis* oocytes after injection of brain mRNA [190] see also [146]. Hill coefficients up to 3.0 were observed for recombinant receptor α subunits expressed in *Xenopus* oocytes [39, 44, 46, 122] or transfected eukaryotic cells [123]. In mouse embryonic spinal cord neurons, the minimum model of receptor activation required binding of 2 and 3 molecules of glycine, respectively, to the two distinct receptors [147]. While binding of two or three glycine molecules is required to activate the receptor ion channel, β-alanine and taurine lack such cooperativity, as deduced from Hill coefficients around unity [122].



Glycine

β-Alanine

Taurine

MDL 27,531

Picolinic acid

Fig. (2). Agonists and potentiators of the glycine receptor.

It has been reported in the literature that binding of strychnine and glycine to the receptor is mutually exclusive. Consistent with this, Hill coefficients of ≈ 1.0 for glycine displacement of [^{3}H]strychnine binding were reported [22, 191-194]. Strychnine inhibition of glycine currents induced in heterologous expression systems was also found to be non-cooperative (n_H ≈ 1.0, [123]). These results are consistent with fully competitive inhibition of glycine-mediated receptor activation by strychnine, i.e. mutually exclusive binding to the receptor of either glycine *or* strychnine. At the same time, a model which assumes overlapping but non-identical binding sites for glycine and strychnine [42, 194, 195] is not excluded, rather supported by structural considerations of amino acid residues contributing to ligand discrimination.

In contrast with these results, Hill coefficients greater than unity were reported for the displacement of [^{3}H]strychnine binding to the inhibitory glycine receptor from rat spinal cord [124]. This observation would require at least partially independent binding sites for both compounds and further suggest a negative allosteric influence of strychnine on receptor agonist binding. A study showing that glycine induces dissociation of [^{3}H]strychnine from the receptor [163] also supports the theory of allosteric modification of the receptor by glycine and strychnine. Glycine-induced dissociation of strychnine [196] is reduced when tyrosine residues essential for glycine binding are chemically modified [124, 163]. Mixed inhibition of the glycine receptor by strychnine was observed in cultured medullary neurons from embryonic rats. Both I_{max} and K_d of the receptor were affected, a result that would also be compatible with close but non-identical binding sites for glycine and strychnine [40]. The conclusion from these results would be that binding of agonist reduces the affinity of the receptor for the antagonist and *vice versa.*

Table 2. Ligand Affinites at the Inhibitory Glycine Receptor

Ligand	K_D or K_i	Species
Amino acid agonists		
Glycine	2-40 µM	rat, mouse
β-alanine	4-57 µM	pig, pigeon
taurine	29-135 µM	
β-aminoisobutyric acid	200 µM	rat
Strychnine and derivatives		
Strychnine	2-14 nM	rat, mouse
Pseudostrychnine	8-10 nM	pig, pigeon
α-Colubrine	12-18 nM	
2-Aminostrychnine	8-23 nM	
Brucine	15-180 nM	
2-Nitrostrychnine	53-500 nM	
Cacotheline	240 nM	rat
N-Methylstrychnine	500 nM 31 nM	rat pigeon
Isostrychnine	37-200 nM	rat
	> 700 nM	pigeon
THIP and analogues		
Iso-THAZ	1.7-6.6 µM	rat
4,5-TAZA	2.8-67 µM	rat
THAZ	20-48 µM	rat
THIP	39-120 µM	rat
β-Spiro[pyrrolidinoindolines]	> 20 µM	rat
Steroids		
RU 5135	3.6-9.8 nM	rat
Pregnenolone sulfate	3.7 µM (EC50)	chick
$GABA_A$ receptor ligands		
Pitrazepin	70-580 nM	rat
(+)Bicuculline	2-7 µM	rat
Bicuculline MeCl/MeI	55-77 µM	rat, mouse
Flunitrazepam	9 µM	rat
Bromazepam, nitrazepam	10 µM	rat
Diazepam	13 µM	rat
PK 8165	4-14 µM	rat
Norhamane	52 µM	rat
Avermectin B_{1a} = Ivermectin	0.8-1.3 µM	rat
Opioids		
Thebaine	900 nM	rat
Sinomenine	23 µM	rat
L-Methadone	30 µM	rat
Alkaloids		
Boldine	67 µM	rat
Gelsemine	18 µM	
Hydrastine	13 µM	
Laudanosine	16 µM	
Vincamine	31 µM	
Miscellaneous agents		
1,5-Diphenyl-3,7,-diazaadamantan-9-ol	27 nM	rat
Imipramine	17 µM	pigeon

Ligand affinities were derived from published displacement data of [^{3}H]strychnine binding as described. See also [227] for references.

Considering these conflicting results, one should keep in mind not only the different experimental conditions and tissue preparations used, but also be aware that competitive and non-competitve inhibition are not easy to distinguish. If a non-competitive, (allosteric) inhibitor binds more tightly to the closed-channel form of the receptor than to the open-channel form, a high concentration of agonist (leading to a higher concentration of open receptor channels) would be expected to overcome receptor inhibition. Phenomenologically, this would result in a right-shift of the dose-response curve, which might be wrongly interpreted as competitive inhibition.

Posttranslational Modification of Receptor Function

Posttranslational modifications, particularly phosphorylation, have been recognized as general regulatory mechanisms of ion channel receptor function [197]. Allosteric modification of neurotransmitter-gated ion channels has been reviewed recently [198]. Receptor modulation by phosphorylation was studied for nicotinic acetylcholine [169], NMDA [199], and 5-HT_3 receptors [170]. Phosphorylation of glycine receptors expressed in *Xenopus* oocytes was shown to be mediated by both protein kinases C and A (PKC and PKA) [200, 201]. Interestingly, glycinergic ligands were found to affect the rate of receptor phosphorylation by PKC. Active (ligand-bound) receptors were found to be preferentially phosphorylated, indicating again the importance of the distinction between active, liganded, and desensitized receptor states [202].

Pharmacology of the Glycine Receptor

Only a few high affinity ligands of the inhibitory glycine receptor have been identified, all of which are convulsants including strychnine and its derivatives, as well as the agent 1,5-diphenyl-3,7-diazaadamantan-9-ol [203], and the steroid RU 5135 [165, 204]. The agonist profile of the receptor is limited to a few ligands outside glycine and related β-amino acids. Recently, some promising ligands were discovered and the combined efforts of biochemical, electrophysiological, and modelling studies begin to shape a picture of the principal sites and forces of interaction of the glycine receptor with its ligands.

A Small Flock: Agonists of the Glycine Receptor

The agonist profile of the inhibitory glycine receptor is, with very few exceptions, limited to glycine and a series of related β-amino acids which mimick functional effects of glycine on motoneuron activity in spinal cord [13, 205], isolated hypothalamic neurons [188], and recombinant glycine receptors [39, 44, 46, 122]. Glycine receptor agonists - ranked by their potency to activate the receptor - are: glycine > β-alanine > taurine > L- and D-α-alanine > L-serine >> D-serine. Detailed analysis using recombinant receptors induced by brain mRNA in *Xenopus laevis* oocytes showed that taurine elicits a smaller maximum current response than glycine [122]. Upon coapplication of both amino acids, taurine partially antagonized the glycine response [206]. This partial agonism, i.e. channel activation, but a reduced maximum current compared to glycine,

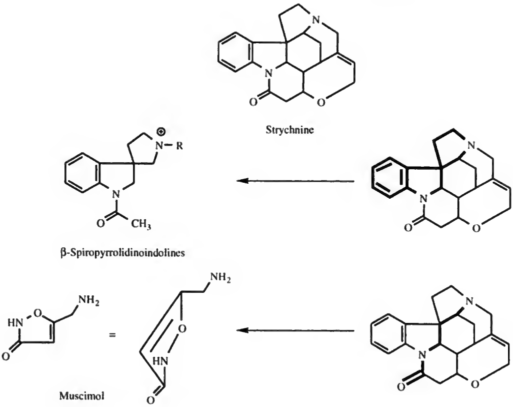


Fig. (3). Strychnine and strychnine-derived antagonists of the glycine receptor.

can be readily explained from the minimum model of receptor activation [182, 184, 207]. For a partial agonist, the channel-open equilibrium constant ϕ is reduced, meaning that at the time of full activation fewer receptor channels are open. If a partial agonist binds to the same site as the 'real' agonist, it should behave as a competitive inhibitor when co-applied with the full agonist.

Some β-amino acids were found to elicit responses in both, inhibitory glycine and $GABA_A$ receptors [206, 208-210]. These findings underline the close structural similarity found for these two receptors. A common structural motif characterizing glycine receptor agonists or antagonists has not yet been identified, although recent modelling studies have made detailed suggestions about the antagonist pharmacophore of the receptor [125, 126].

Partial agonism by β-amino acids shows that the size of the glycine molecule does not impose any steric restrictions on agonist binding. Yet despite structural similarities to glycine, larger compounds such as substituted heterocycles including aminohydroxyisoxazoles and pyrazoles are inactive at the glycine receptor [211].

Strychnine, the Principal Inhibitor of the Glycine Receptor

Strychnine

Strychnine is an indole alkaloid isolated from the seeds, bark and roots of the Indian tree *Strychnos nux vomica* and other members of the *Strychnos* plant family (Longaniaceae). It was first isolated in 1818 by Pelletier and Caventou [212, 213]. Its total synthesis was achieved by Woodward in 1954 [214].

Strychnine was originally used as a rodenticide and is still in use in Europe [215], prescription in the USA was discontinued in 1985 [216, 217]. Its low lethal dose of about 1 mg/kg in man [218] have made it a popular compound in both reality and fiction to settle personal and financial differences, or improve personal rankings within a heritage line.

Strychnine intoxication is characterized by hyperreflexia and altered tactile, visual and acoustic perception. Sensory stimulation initiates painful convulsions, and arrest of respiration which can cause immediate death [217, 218]. The course of strychnine poisoning has been described [212, 219]. Loss of glycinergic inhibition can be overcome by treatment with diazepam [220, 221], or other $GABA_A$ potentiators. Likewise, hyperekplexia can be treated to an extent with drugs that increase $GABA_A$ receptor activity. Clonazepam, which potentiates $GABA_A$ receptor function can reduce the startle activity in hyperekplexia, while stiffness was unaffected. Vigabatrin, an inhibitor of the catabolic, GABA-removing enzyme GABA transaminase, was without effect [222].

The strong and highly selective interaction of strychnine with the inhibitory glycine receptor has been mentioned before. Indeed, only at higher concentrations does strychnine detectably interfere with non-glycinergic receptors and channels (reviewed in [223], but see [224]). From a comparison of the size of glycine and strychnine molecules, it is certainly not immediately obvious that both ligand should not only interact with the same receptor, but even compete for overlapping or even identical binding site. Yet the use of nanomolar concentrations of [^{3}H]strychnine as a reliable and highly specific probe of the glycine receptor [124, 225] is well established, and affinity chromatography on 2-aminostrychnine columns has been used to purify glycine receptor from mammalian spinal cord [10-12, 48].

Glycine-receptor agonist potency of partial agonists parallels their ability to displace [^{3}H]strychnine binding to synaptic membranes from mammalian spinal cord [124, 160, 225]. Glycine displays the highest receptor affinity ($K_i \approx 10$ - 30 μM), β-amino acids have lower affinities decreasing in the order of β-alanine > β-aminoisobutyric acid > taurine. GABA and higher ω-amino acids are without significant effect [19, 20, 22, 160, 225, 226].

Strychnine Derivatives and Fragments

The strychnine molecule has been subjected to a number of structure-activity studies. While a part of its structure can be aligned with the glycine molecule, enabling interaction with the ligand binding site, the bulk of the molecule is thought to block the ion channel thereby inhibiting ion channel flux. The effects of substitutions and modifications of the strychnine molecule have been discussed in detail [227].

A group of β-spiro[pyrrolidinoindolines] represent structural fragments of the strychnine molecule, conserving its indole moiety (ring I and II), the pyrrolidine ring V, and amide group on N-9. Convulsant activity and glycine receptor affinity ($K_i \geq 20$ μM) of these compounds closely correlate, and agonistic β-spiro[pyrrolidinoindolines] are not known [228]. Receptor affinity critically depends on the substitution of the two nitrogen atoms in position 9 and 19 of the spiro[pyrrolidinoindoline] structure. A secondary amine in position 19 reduces affinity towards the glycine receptor about tenfold, while removal of this nitrogen, as in spirocyclopentylindoline, results in an affinity of > 100 μM [228]. Convulsant activity of β-spiro[pyrrolidinoindolines] also depends on the acetyl group on the indole ring nitrogen in position 9. Removal of this acetyl group causes a pronounced loss in receptor affinity ($K_i = 260$ μM) [228].

Non-Strychnine Glycine Receptor Antagonists

A wide variety of synthetic and natural glycine receptor antagonists has been presented and their pharmacology discussed [227]. The reader is directed to this review for additional reference; recent developments will be presented in the following paragraphs.

Synthetic Glycine Receptor Antagonists

The amidine-derivative 1,5-diphenyl-3,7-diazaadamantan-9-ol is a potent convulsant [229, 230] which efficiently antagonizes glycine- but not GABA-mediated neuronal inhibition [205]. It was found to be a high affinity inhibitor of [^{3}H]strychnine binding to the glycine receptor (K_i 27 nM) [203]. The agent also appears to interfere with central nicotinic cholinergic synapses [205]. Its chemical structure is reminiscent of *tert*-butylcyclophosphorothionate (TBPS), a "cage" convulsant at the $GABA_A$ receptor [231, 232]. However, the mechanism of glycine receptor inhibition by 1,5-diphenyl-3,7-diazaadamantan-9-ol is not known.

Several benzimidazole derivatives were tested as potential glycine antagonists. However, only K_i values in the μM range (5.3 μM and higher) were found for this class of substances [233].

4-Hydroxyquinolines and 4-hydroxyquinoline-3-carboxylic acids were found to be antagonists of the glycine receptor [234]. This is a remarkable finding, since these compounds are structurally closely related to 2-carboxy-4-hydroxyquinolines, which are antagonists of glycine binding to its co-agonist site on the NMDA receptor [234]. While many examples of cross-inhibition and activation between glycine and GABA receptors are known, this is the first observation of a chemical link between glycine binding sites on glycine and NMDA receptors. Thus, there might be a structural similarity between the glycine sites on the both receptors - it remains to be seen if future research will provide insight into the atomic arrangement in the binding pockets of both receptors.

Effects of some alkaloids and opioids have been discussed [71]. Inhibition constants in the micromolar range are usually observed for these substance classes.

Convulsant Steroids

One of the most powerful antagonists of glycine-mediated inhibition in spinal cord neurons is RU 5135 (3α-hydroxy-16-imino-5β-17-aza-androstan-11-one), a convulsant amidine steroid [235]. This steroid is characterized by the highest affinity known ($K_i \approx 5$ to 13 nM) of any glycine receptor ligand [127, 163, 165]. It is, however, not entirely specific for glycine receptors and also antagonizes $GABA_A$ receptor ligand binding [204]. RU 5135 and strychnine share some thermodynamic aspects of binding to the glycine receptor. Receptor binding is enthalpy-driven [127], and enhanced by sodium chloride [165], suggesting that the agent recognizes the strychnine binding site of the glycine receptor. This conclusion is, however, in contrasted by observations with other steroids including progesterone, deoxycorticosterone, and pregnenolone sodium sulfate which also suppress neuronal glycine responses at micromolar concentrations. In the case of the neurosteroid pregnenolone sulfate, inhibition of glycine receptor-mediated responses in cultured chick spinal cord neurons was not voltage- or agonist-dependent. The dose-response curve in the presence of pregnenolone sulfate was shifted to the right, so that competitive, non-channel-blocking inhibition of the glycine receptor was postulated [236]. It was previously suggested that these steroids act through a glycine receptor domain distinct from the strychnine binding site [237]. It thus remains to be shown whether RU 5135 and progesterone-like steroids recognize identical or distinct glycine receptor domains. It should be noted in this context that progesterone and other steroids are positive modulators of the $GABA_A$ receptor, [238].

Using molecular modelling techniques, two groups of glycine receptor antagonists with different modes of action were suggested. Group 1 (strychnine, brucine, pitrazepine and biccuculine methobromide) interacts with the receptor through electrostatic forces, but the open channel induced by ligand binding is covered or blocked by a part of the molecule itself

[125]. Group 2 antagonists (R5135 and 1,5-diphenyl-3,7-diazaadamantan-9-ol) bind to the receptor electrostatically but do not induce channel-opening [125]. This interpretation is in agreement with findings that suggest partially overlapping sites for glycine and strychnine on the glycine receptor [128].

GABA Receptor Ligands

Structurally, the inhibitory glycine receptor is highly homologous to the $GABA_A$ receptor. This homology extends to functional characteristics including conductance properties of the intrinsic ion channels [138, 144], ion dependence [162] and kinetics [239] of ligand binding. In addition, a wide spectrum of ligands recognizes both receptors. Similarities in agonist (β-amino acids) binding between inhibitory glycine and $GABA_A$ receptors were reported [206, 208-210].

Muscimol Analogs

In a series of structure activity studies on heterocyclic muscimol derivatives, Krogsgaard-Larsen and coworkers [240] have identified structural determinants of ligand specificity for glycine and $GABA_A$ receptors (see also [71]).

The mode of binding of strychnine and 8 muscimol derivatives to the glycine receptor was analyzed by molecular modelling techniques [126] using a pharmacophore for the binding site that was proposed earlier by the same group [125]. All antagonists possess three charged groups necessary to interact with the ligand-binding pocket of the receptor and a fourth positive charge that can block the chloride channel. However, only strychnine has a structure that allows for an extra interaction of its fourth positive charge with a tertiary carboxylic group on the receptor. This bidentate interaction is responsible for the strong interaction and potent receptor inhibition by strychnine [126]. Molecular biological data confirm the presence of the negative charge required for this interaction. Even though modelling approaches critically depend on the right choice of parameters and may not be able to fully accomodate local hydration and conformational flexibility, these results provide a useful background for further structure-activity studies.

Non-selective $GABA_A$ Receptor Ligands

The convulsant alcaloid bicuculline, a $GABA_A$ receptor antagonist [189, 241, 242], was found to inhibit glycinergic transmission in cat cuneate neurons [243, 244]. The more active (+)-isomer of bicuculline displays micromolar K_i-values at the glycine receptor [128, 165, 245]. Bicucculine prevents $[^3H]$strychnine binding at concentrations where glycine responses of the receptor are not yet affected. It was suggested from protein modification studies, ionic and temperature effects, that bicucculine acts as a strychnine-like antagonist of the glycine receptor [127, 128, 165].

However, glycine responses in spinal cord neurons [241], in channels induced by brain mRNA in *Xenopus laevis* oocytes [206, 210, 246], and in recombinant glycine receptors expressed in a human cell line [123] appear to be unaffected by bicuculline. Thus, bicucculine can not be considered a specific glycine receptor probe; the source of the receptor protein, and possibly its developmental status must not be ignored.

The compounds pitrazepine, SR95103 as well as benzodiazepines and barbiturates are all $GABA_A$ receptor antagonists. They also show some activity at the glycine receptor, but these interactions usually require micromolar concentrations [227]. It has to be borne in mind that the GABA receptor is the primary pharmacological target of these substances. The same applies to the anthelmintic ivermectin. In this case, however, interaction with the inhibitory glycine receptor is complex [247]. It has been suggested that the agent interacts with as yet unidentified regulatory sites on both, glycine and $GABA_A$ receptors [227].

Picrotoxin, another blocker of the $GABA_A$ receptor channel, efficiently antagonizes glycine-gated currents, even though it only poorly inhibits $[^3H]$strychnine binding to the receptor. Current responses induced in heterologous expression systems are almost completely abolished by picrotoxin concentrations [72, 122, 123] which fail to displace $[^3H]$strychnine binding [247]. These conflicting findings are further complicated by the observation that two kinetically distinct phases of glycine receptor-mediated whole-cell currents on cultured embryonic mouse spinal cord cells were differently affected by picrotoxin [147]. It has been suggested that picrotoxin *in*sensitivity is conferred to the glycine receptor through the β subunit [248]. Thus, developmental regulation [36, 72] might lead to glycine receptor populations with non-uniform picrotoxinin sensitivity. This observation could also reconcile the conflicting reports that glycine-mediated inhibition of spinal interneurons [249] and cuneate neurons [244] appears to be susceptible to picrotoxin, while no sensitivity is seen in the oculomotor nucleus [250], isolated spinal cord of immature rats [251], or cultured neurons [189]. Additionally, picrotoxin might interact with an allosteric site on the receptor, and only at higher concentration would it be expected to bind to the strychnine site.

Corymine, a plant alkaloid from the leaves of *Hunteria zeylanica* was found to potentiate convulsions induced by both picrotoxin and strychnine. It inhibited glycine responses in *xenopus* oocytes injected with rat spinal cord RNA more potently than GABA responses [252].

The Hope Chest of Glycinergic Drug Design: Positive Modulation of Receptor Function

Defects or malfunctions of the glycine receptor, leading to loss of glycinergic synaptic inhibition, cause motor dysfunctions that resemble strychnine intoxication. These symptoms can be alleviated by treatment with substances that increase inhibitory synaptic transmission. To date, potentiators of $GABA_A$ receptor channels are most commonly used, as selective enhancers of glycine receptors are not available. Naturally, higher CNS functions, where $GABA_A$ rather than glycine is thought to be the predominant inhibitory neurotransmitter, are also affected under therapy. While selective positive modulators of the glycine receptor are currently not available, some encouraging results were recently obtained.

Chances for Agonist Derivatives?

It is of interest to note that although glycine and $GABA_A$ receptors are structurally related, they are highly selective for their respective neurotransmitters. Nevertheless, both receptors respond to the same β-amino acids. In isolated rat optic nerve and cuneate nucleus [208], cultured chick spinal neurons [209], and *Xenopus laevis* oocytes injected with brain messenger RNA [206, 210, 246], β-alanine and taurine displayed pharmacological and cross-desensitization properties compatible with a dual action on both receptors. Taurine and β-alanine responses on either receptor are sensitive to both, the $GABA_A$ receptor antagonist bicuculline and the glycine receptor antagonist strychnine [206, 253]. This observation is somewhat qualified by a decrease of receptor selectivity of these convulsants at the micromolar concentrations employed [249].

Analyzing the effects of a series of β-amino acids on recombinant glycine receptors, it was concluded that the cis-conformation mediates agonistic behaviour, while the trans-configuration confers antagonistic properties on these compounds. Consistent with this hypothesis, nipecotic acid, having a fixed trans configuration, was found to behave as a purely competitve antagonist of the glycine receptor [254].

These findings are in agreement with earlier studies [188], which suggested that β-alanine and taurine can assume extended and folded conformations which allow their amino and carboxyl functions to assume an orientation resembling glycine. The dual effect of these β-amino acids on glycine and $GABA_A$ receptors [206, 208-210] was proposed to result from a transition between these conformations.

New Agonists? - Picolinic Acid

Picolinic acid was shown to delay onsets of strychnine-induced seizures, this muscle relaxant and anticonvulsant activity being due to inhibition of spinal glycinergic neurons [255]. Indeed, low-efficiency strychnine displacement by picolinic acid and 6-aminonicotinic acid has observed, estimated displacement constants were in the submillimolar range (C.-M. Becker, unpublished results). This is the first observation of a glycine receptor agonist that is *not* structurally related to glycine and it might lead to a new class of full or partial glycine receptor agonists.

Potentiators of Glycine Receptor Function

Potentiation of glycine- and taurine-mediated currents in the presence of volatile anaesthetics was reported [256]. Potentiation was on the order of 20 %, and was higher for recombinant receptors than in the case of medullary neurons [256]. A similar potentiation of glycine receptor function by n-alcohols and anaesthetics was reported; the length of the carbohydrate chain of the alcohol was found to affect potentiation. Up to 10 carbon atoms produced robust (150 - 200 %) potentiation of glycine-mediated currents, while potentiation by higher alcohols was less (30 - 60 % for dodecanol). The 'cutoff' for glycine receptor potentiation by *n*-alcohols depends on amino acid residues within transmembrane segments 2 and 3 of the α subunit, particularly at position α267 [257]. Ethanol was found to enhance glycine receptor function. This effect was found to be directly correlated to the molecular volume of the amino acid residue at α267 [258].

Anaesthetics, in general, are effective only at higher concentrations (i.e. where anaesthesia would be expected) [259]. The anaesthetic propofol, however, was found to enhance GABA- and glycine-mediated current on recombinant glycine receptors expressed in *Xenopus* oocytes. For glycine receptors, an EC_{50} of 27 µM was found, glycine-mediated currents were increased by some 30 %. At higher concentration, propofol itself activated glycine and GABA receptors [260]. In this study, potentiation was also seen with pentobarbitol and trichloroethanol, the major metabolite of chloral hydrate.

Zn^{2+} ions in nanomolar concentrations were found to enhance glycine receptor function [261]. As described earlier, this modulation was biphasic, and higher concentrations of Zn^{2+} attenuated glycine receptor function.

Penicillin was found to inhibit glycine-activated whole-cell currents when co-applied with the agonist. However, when glycine was applied immediately after preincubation with penicillin, glycine currents were potentiated [262]. Although remarkable, this effect might be difficult to exploit therapeutically.

Adrenoreceptors α1 and α2 were found to indirectly potentiate taurine-mediated whole-cell currents in acutely isolated neurons from rat substantia nigra [263, 264]. This effect of α1 and α2 receptors was mediated through protein kinases A and C, respectively.

A striking effect was seen with MDL 27,531, which blocked strychnine-induced seizures in mice with ED_{50} values of 7.3 mg/kg (oral) and 12.8 mg/kg (intraperitoneal) [265]. The blockage was highly selective: MDL 27,531 was without effect against the GABA antagonist bicucculine, quinolinic acid and mercaptopropionic acid. Strychnine antagonism by MDL 27.531 was observed at concentrations where no sedation, motor ataxia, muscle relaxation or CNS depression occured [265].

Indirect Modulation of Glycinergic Function: Glycine Uptake

Since carriers of small neutral amino acids are missing on the luminal side of the blood-brain barrier, efforts were made to synthesize lipophilic derivatives of glycine, β-alanine and GABA by N- or O-substitution. The agonist (or antagonist) properties of these derivatives were, however, not fully described [266]. The distribution of glycine transporter isoforms has been discussed earlier. By analogy to the powerful effects of serotonin reuptake inhibitors (such as fluoxetine), glycine uptake might prove a useful therapeutical target for modulation of receptor function.

Pharmacology of Receptor Phosphorylation: A Therapeutic Window?

Glycine-mediated whole-cell currents of homomeric α1 receptors expressed in *Xenopus* oocytes were decreased when PKC activators were applied, suggesting an involvement of PKC in receptor negative regulation. The intracellular amino acid residue serine 419 was proposed as phosphorylation site [267].

Another study of the effects of PKC on homomeric $\alpha 1$ and $\alpha 2$ glycine receptors expressed in *Xenopus* oocytes showed that phosphorylation by PKC also reduced glycine-mediated currents [268]. Phorbol esters may have a direct effect on the glycine receptor in addition to their PKC activating function [268]. In acutely dissociated hypothalamic neurons, glycine receptor phosphorylation by PKA reduced the rapidly desensitizing glycine current phase [269].

In contrast, glycine receptors on rat hippocampal neurons showed a 2.6-fold *increase* in current response when phosphorylated by PKC [270]. Likewise, glycine currents in acutely isolated rat dorsal horn neurons were enhanced when the alpha subunit of CaM-KII (Calcium/calmodulin-dependent protein kinase II) was intracellularly applied [271]. 2,3-Butanedione (BDM) modified glycine currents on murine hypothalamic neurons [272]. In this case BDM seems to activate PKC while at the same acting as a chemical phosphatase of the receptor.

It should be noted in this context, that the glycine-receptor associated protein gephyrin is also phosphorylated by an endogenous kinase activity which was shown to be unaffected by activators or inhibitors of cyclic nucleotide-dependent kinases, Ca^{2+}/calmodulin-dependent kinases, or PKC [273]. Despite its almost ubiquitous occurence in the body, phosphorylation might offer a chance - albeit faint - of indirect control of glycinergic function.

Conclusions

Prospects for a Therapeutic Orphan?

The components of glycinergic signal transmission are being investigated and understood in impressive and ever-increasing detail. Elucidation of genetic structure and localization have allowed to diagnose hereditary disorders of the glycine receptor. From a knowledge of receptor topology and basic pharmacology, protein regions and individual amino acids that confer specific properties on the receptor have been identified, and attempts to model the ligand-binding site of the receptor have made it possible to consider the functionalities involved in receptor-ligand interactions.

Yet, despite all this detailed knowledge, compounds that allow an effective control of glycinergic transmission, and hence the ability to treat the symptoms of hereditary receptor defects, are still lacking.

Nonetheless, recent developments have shown that compounds exist which selectively augment glycine receptor function. To date, all therapeutical candidates have moderate potency and selectivity, but may still be the first step towards at effective treatment of glycine receptor-mediated diseases.

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Therapeutic Approaches to the Treatment of Neuroinflammatory Diseases

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Abstract: Microglia cells are present in the central nervous system and respond quickly to pathogenic stimuli in order to protect the brain. When these immunological responses activate inappropriately or are prolonged, they can contribute to the neuronal damage observed in many neurodegenerative diseases. A variety of immune system modulators including complement proteins, inflammatory cytokines such IL-1α, IL-1β, IL-3, IL-6, TNF-α, and S100β, colony-stimulating factor-1, coagulation proteins and matrix metalloproteases are made by both microglia and astrocytes. Additionally astrocytes, the predominant glial component of the brain, express cell-adhesion molecules, cytokine receptors and induce nitric oxide synthease. The pathophysiology of Alzheimer's disease, stroke, traumatic brain injury, and multiple sclerosis suggest that a large portion of the irreversible damage observed can be attributed to a neuroinflammatory mechanism. The immunomodulators of these diseases are reviewed and new agents within specific molecular mechanisms are presented and discussed.

Introduction

Compared to other organs in the body, immunological reactivity in the central nervous system is markedly reduced and almost undetectable [1]. Additionally, the existence of the blood brain barrier, the absence of conventional lymphatic drainage, and the unusual tolerance of the brain to transplanted tissue have reinforced the idea that the brain is somehow isolated from immunological processes [2]. Recent evidence derived from advanced methodology such as immunohistochemistry and molecular biology now indicate that the brain is neither immunologically privileged nor isolated. In fact the brain appears to be immunologically vulnerable due to the fragility of neurons and their complex interconnections. The immune system is intended to safeguard mammalian hosts from invading microorganisms. However, in the brain, any response to an immunological challenge may place host tissue at risk. This is because neurons are post-mitotic cells with no ability to divide and little ability to recover from injury. Therefore, immunological functions that would have little deleterious effects on many organs of the body, can have serious consequences for the brain. Neuroinflammation is a silent process differing from peripheral inflammation in that some of the cardinal signs (tumor, dolor) do not exist, while others (rubor, calor) cannot be evaluated [3].

Three major cell types are involved in inflammatory processes in the central nervous system. These include the endothelial cells, the astrocytes and the microglial cells [1]. Without question, the microglial cells are the primary safeguard of the brain. These cells are thought to be derived from bone-marrow precursors that invade the brain at an early state of embryonic development and reside there as tissue-specific macrophages. They comprise about 10-20% of the total glial population [4] and are distributed randomly throughout the brain with some prevalence in gray matter, cerebral cortex, hippocampus and in lower numbers in dorsal thalamus. No brain region is without microglia which ensures quick responses when other cell types are compromised [5,6]. Microglia normally are present in the brain in a quiescent or resting phase. While molecular mechanisms which trigger the production and release of microglial growth factors are for the most part unknown, the

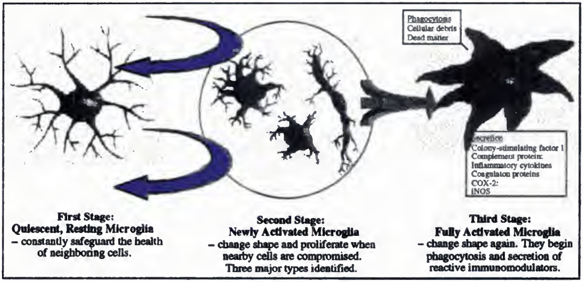
Phagocytosis
Cellular debris
Dead matter
Secretion
Colony-stimulating factor 1
Complement proteins
Inflammatory cytokines
Coagulaton proteins
COX-2:
iNOS
First Stage:
Quiescent, Resting Microglia
– constantly safeguard the health of neighboring cells.
Second Stage:
Newly Activated Microglia
– change shape and proliferate when nearby cells are compromised.
Three major types identified.
Third Stage:
Fully Activated Microglia
– change shape again. They begin phagocytosis and secretion of reactive immunomodulators.

Fig. (1). Activation of microglia.

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resident microglial cell probably become activated when dying or damaged cells emit chemical signals (Fig. (1)). Microglia activation typically occurs locally and can be characterized in the early stages by proliferation and changes in cell shape. Altered ion fluxes through ligand and voltage-gated K^+-channels may induce expression and subsequent production of microglial growth factors by other brain cells or by the microglia themselves [1]. In the microglia's differentiation phase, the cells release mediators of inflammation and begin to actively phagocytose dying cells and debris. Microglia cells may also migrate to cell bodies of injured neurons where they reportedly interrupt synaptic contacts as a means of allowing damaged neurons to recover from injury and regenerate. If some of these microglia remain activated after a potential threat has ceased, their positive function may change into a health threatening role as observed in some chronic neurodegenerative diseases [1].

There is a growing list of immune system proteins that have been found to be produced by brain cells [7]. For example, they can produce complement proteins [8,9], inflammatory cytokines, coagulation proteins, and such toxic products as the membrane attack complex (MAC) of complement and superoxide radicals. A variety of inflammatory cytokines, including interleukin-1α (IL-1α), interleukin-1β (IL-1β) [10-12,8], interleukin-3 (IL-3), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) [13] and colony-stimulating factor-1 are made by both microglia and astrocytes [2]. Additionally, astrocytes, the predominant glial component of the brain have been shown to produce major histocompatibility complex class I and class II proteins, express cell-adhesion molecules and cytokine receptors [4] and induce nitric oxide synthease (iNOS) [14].

While it is outside the scope of this review to comprehensively discuss the pathophysiology of all neurodegenerative diseases that possess a neuroinflammatory component, this article will focus on the primary immune components of three major neurodegenerative diseases, namely Alzheimer's disease (AD), stroke and multiple sclerosis (MS). In each case, the immune function most closely associated with the disease will be discussed and new agents within a molecular mechanism will be emphasized as possible future therapeutic interventions.

Alzheimers Disease

One of the classical hallmarks of Alzheimers disease are the amyloid plaques that are prevalent in the diseased brain at autopsy. Activated microglia are densely embedded in senile plaques and have gathered around neuronal cell bodies, neurofibrillary tangles, and dystrophic neurites [15,16]. Inflammation is unlikely to be an etiologic cause of AD nor is it likely to be the only pathogenic mechanism of the disease. There is, however, substantial evidence that inflammation is an important contributor to AD pathology and the neurodegeneration that underlies AD dementia. Inflammatory reactions would appear to be sufficient on their own to cause significant damage to the AD brain [15]. Although the specific signals, resulting in microglial activation remain to be resolved, it is possible that signals activating microglia in AD are distinct from signals activating microglia in HIV-infections or MS.

In recent studies, the relationship between microglial activation and plaque evolution was examined using tissue at autopsy from AD patients and non-demented control subjects. One striking difference between senile and diffuse deposits is the presence of activated microglia and reactive astrocytes in the vicinity of the former, but not the latter. Upon close examination, a preferental association of primed microglia with diffuse amyloid-β deposits can be observed and implies that microglial transformation from primed to enlarged to phagocytic types occurs in concert with the evolution of amyloid plaques from diffuse deposits to the neuritic amyloid-β plaque forms seen in AD. Microglial phagocytic activity may reflect involvement in the processing of diffuse amyloid into condensed amyloid-β or in clearance of neuritic debris [17,18]. Reactive astrocytes can be seen to surround the perimeter of the senile plaque, as if to wall it off, and send processes into the plaque to contact the amyloid-β [19]. Microglia and astrocytes are, thus, appropriately situated to mediate the neuropathology that occurs in the vicinity of senile plaques. Both these glial cells can be activated directly by fibrillar amyloid-β within senile plaques and are not merely reacting to the presence of degenerating neurites [7].

The Complement System

One of the body's major immunological responses to invading pathogens is the classical complement system. This classical pathway is normally initiated by the binding of C1q protein to an antibody-antigen complex and mediates attack by multiple mechanisms. The complement system provides signals for scavenger activation, opsonization, and direct lysis of cells by opening holes. The inflammatory response in AD brain, which involves vigorous activation of the complement system, does not involve antibodies, indicating that the irritant action is

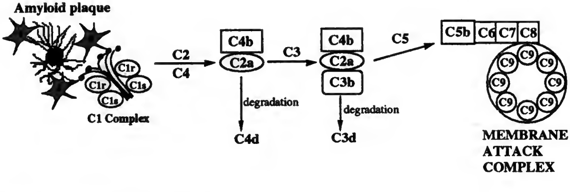


Fig. (2). Activation of the classical complement cascade by amyloid-β.

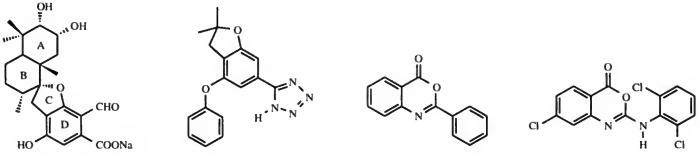
not of the classical autoimmune variety. In support of this observation, there does not appear to be an appreciable quantity of immunoglobulins in the vicinity of senile plaques, yet the complement system is known to be activated in patients with AD. This raises the question as to how the complement cascade is initiated in AD. Several laboratories have now shown that amyloid-β peptide can bind C1q and function as a potent activator of the classical complement pathway (Fig. (**2**)). [7,2,21]. Electron micrographs of complement immunoreactivity in the vicinity of amyloid-β deposits demonstrate that axons are under active and effective complement attack. The terminal product of the classical complement cascade, the membrane-attack-complexes (MACs) which insert into plasma membranes with resulting cell death, have been demonstrated on the dystrophic neurites surrounding the plaques [22,23]. The observed endocytosis also strongly suggests that these neuronal cells are not already existing AD detritus, but are living cells with axons under complement attack [15]. Activation of the complement cascade may have secondary effects as well with increased levels of certain complement proteins are likely to contribute and amplify immune mechanisms of pathogenesis. Studies show that C1q may accelerate the aggregation of amyloid-β and the cycle continues since the more aggregated amyloid-β that exists, the better it activates C1q [15].

There have been several efforts to intervene in this pathological cascade and these have been recently reviewed [24]. In one of the first attempts to explore the interactions of small molecules with complement, K-76COONa **1** was observed to inhibit the classical pathway at the C5 activation step. Compound **1** inhibits anaphylatoxin C5a production with 50% maximal inhibition (IC_{50}) of 3 mM [25]. A series of C/D-ring analogs of **1** were synthesized and a tetrazole analog **2** significantly improved the human complement inhibitory potency with an IC_{50} of 195 μM [26]. Researchers as Parke-Davis have described several series of benzoxazinones which are potent inhibitors of C1r, one of the early serine proteases in the classical pathway. The 2-aryl and 2-anilino-benzoxazinones **3** and **4** are representative of these compounds with IC_{50}s of 1.4 and 0.4 μM respectively [27,28]. Many of the approaches which block complement activation by inhibiting the enzymatic activity of C1r, C3 or C5 convertase complexes would presumably inhibit complement damage seen in AD, but would compromise the complement system throughout the body. The demonstration that amyloid-β causes complement activation suggests that it should be possible to identify molecules that bind to amyloid-β and block initiation of the classical complement pathway without affecting the normal antibody-mediated cascade. Proof-of-concept has been demonstrated by researchers at Gliatech when a peptide comprised of a portion of the C1q A chain sequence (A-G-R-P-G-R-R-G-R-P-G-L-K) was shown to effectively inhibit amyloid-induced complement activation, but had little effect on the complement activation mediated by aggregated human IgG. Human IgG is believed to bind a different sequence of C1q for activation [7]. The challenge now remains to retain this activity with a small bioactive molecule.

Cyclooxygenase Inhibition

In addition to the pharmacological-driven quest for new drugs to treat AD, the epidemiologists have provided a body of evidence that suggests that antiinflammatory agents may delay the onset and slow the progression of AD. Seventeen epidemiologic studies have been published which include antiinflammatory drugs or arthritis as a risk factor for AD. The most logical explanation for the observed negative association between arthritis generally and AD is the long-term use of antiinflammatory drugs. Nonsteroidal antiinflammatory drugs (NSAIDs) are by far the most commonly used agents for these conditions. The results from these studies have been recently reviewed [3]. In one recent study, the increasing duration of NSAID use (including ibuprofen, naproxen, indomethacin, fenoprofen and flurbiprofen) in patients with a variety of ailments was associated with a decreasing risk of AD. No similar association was found with use of acetaminophen which possesses no antiinflammatory activity. Surprisingly, no association was noted between aspirin and risk of AD [29]. This points to the fact that unfortunately, at present, the data are too uncertain to predict which drugs or combination of drugs might be useful. Only limited data exist on the relative ability of various NSAIDs to cross the blood brain barrier. To date there has been only one clinical trial testing NSAID action as antiinflammatory therapy for AD. This was a small, double-blind placebo-controlled pilot study of six months duration using the NSAID indomethacin. The patients on drug appeared to stabilize while patients taking placebo had a deterioration at a rate comparable to that observed in other studies [30]. More comprehensive trials are ongoing to verify whether these epidemiologic data genuinely reflect a protective effect of antiinflammatory drugs for AD [30].

NSAIDs are inhibitors of cyclooxygenase (COX) which oxidize arachidonic acid to prostaglandins (PGs). In recent years, two forms of this enzyme have been identified. COX-1 is a



1 2 3 4

Fig. (3). Inhibition of the complement cascade.

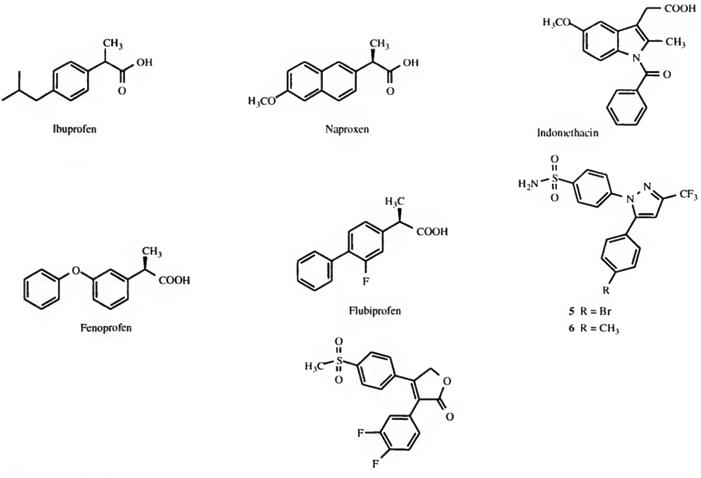


Fig (4). Cycloxygenase inhibitors.

constitutive form which is necessary for the health of certain cell types and a second form, COX-2 is inducible and is thought to be responsible for the high production of prostanoids associated with inflammatory response. The expression of COX-2 has been recently investigated in rat primary microglial cultures. Gene transcription is differently regulated in response to lipopolysaccharide and the transcription factor, NF-κB seems to largely control expression of COX-2 and other inflammatory mediators in activated microglial cells [4]. Classic NSAIDs (nonselective COX inhibitors) generally suffer the side-effects of decreased renal blood flow, decreased platelet function, dyspepsia and gastric ulceration [31-33]. Recently, selective COX-2 inhibitors have been developed and have demonstrated antiinflammatory [32] and analgesic activity without causing the gastrointestinal damage associated with current non-selective COX inhibitors [31,33]. Mechanistically, selective COX-2 inhibitors decrease inflammation by preventing overproduction of PGs while allowing COX-1 to produce the basal levels of PGs necessary for the health of certain tissues most notably the gastrointestinal tract [31]. The realization that the serious side-effect profile can be markedly reduced has led the pharmaceutical industry to expend much effort to discover selective COX-2 inhibitors for the world market. Recently, the unliganded crystal structure of murine COX-2 and complexes with flurbinprofen, indomethacin and SC-558 **5**, a selective COX-2 inhibitor have been published [34]. A pharmacophore model has also been developed and reviewed [31]. While there is no selective COX-2 inhibitor currently marketed, several of these new drugs are in phase three clinical trials for arthritis and pain indications. Two selective COX-2 inhibitors, Celecoxib **6** from Searle and MK966 **7** from Merck have been reported to be entering clinical trials for AD in the near future. Large studies backed by the drug industry may provide definitive studies regarding the value of this important class of drugs for the treatment of AD.

If NSAIDs are used to ameliorate the disability from AD then it is logical to assume that steroidal antiinflammatory agents would have a place in the medicinal arsenal against this disease. Steroids readily cross the BBB, but the long-term consequences of the use, particularly in the elderly is problematic. Steroids will damage hippocampal neurons in culture [35] and in AD such neurons are already among the most vulnerable to the disease. In vivo, steroids produce electrolyte disturbances that induce congestive heart failure, osteoporosis leading to bone fractures, and vulnerability to infection [3,35]. Despite these drawbacks, a preliminary trial of low-dose prednisone demonstrated that this treatment was well-tolerated, but provided no suggestion that the drug had any palliative or ameliorative effect in AD. A larger treatment trial with prednisone is now being conducted by the Alzheimers Disease Collaborative Study Group [36].

The finding that NSAIDs ameliorated or prevented the process of AD whereas steroids did not, suggests that COX may be mediating neuronal damage by mechanisms that do not include classical inflammatory pathways [37]. For example, COX is an important mediator of signal transduction in the calcium-dependent postsynaptic cascade that follows glutamatergic stimulation of the pyramidal cells with N-methyl-

D-aspartate (NMDA) type receptors [38]. NMDA receptor activation is present to induce memory and learning by long-term potentiation, but in pathological states activation can result in excessive stimulation and excitotoxic cell death [39,40]. An alternative target for COX inhibition may be the blockade of synthesis and/or release of PGs by astrocytes in response to IL-1 [37]. Additional studies to clarifiy these mechanisms are ongoing.

Immunosuppressive Therapy

Autoimmune reactions are present in every case of AD. This suggests that immunosuppression would be a reasonable therapeutic strategy [41]. Steroids are not good candidates for the reasons cited above, but cytostatic drugs used in cancer chemotherapy have exhibited potent immunomodulatory activities. Cyclophosphamide treatment in moderate, immunosuppressive doses was tested within a group of 32 AD patients. A marked improvement as compared to the control group of patient was observed as measured by a Mini Mental State (MMS) examination [42].

Neuronal Cytokines

Other inflammation-related proteins have been demonstrated to exacerbate AD pathology. Several studies have shown that amyloid-β stimulates cytokine production [43] which may in turn increase amyloid precursor protein (APP) production [44-47]. The pattern of microglial IL-1α immunoreactivity across brain regions in AD mirrors the regional distribution of the amyloid-β derived neuritic plaques. Microglia-derived IL-1 may contribute to astrocytic actions occurring within the evolving amyloid plaques [48]. Additionally, IL-1β upregulates APP expression in vivo in rat brain. This supports the idea that the overexpression of IL-1α and IL-1β in AD induces excessive synthesis and processing of APP [49]. These neurotrophic APP fragments could stimulate further growth with consequent demand for synthesis of APP [50].

The activated astrocytes associated with the neuritic plaques of AD also overexpress the neurotropic cytokine S100β. The correlation between the density of S100β-immunoreactve astrocytes and neuritic plaques in cerebral regions in AD implicates this cytokine in the growth of dystrophic neurites in amyloid-β plaques. The close association of IL-1α microglia and S100β immunoreactive astrocytes in neuritic plaques suggest that IL-1α may be the in vivo stimulus that induces astrocytic overexpression of S100β [51].

Using immunohistochemical methods, the presence of IL-6 has been demonstrated in plaques in the brains of AD patients while plaques in brains of nondemented elderly did not exhibit IL-6 immunoreactivity [52]. The fact that IL-6 can be found in plaques where neuritic pathology has not yet developed suggests that the appearance of IL-6 may precede neuritic changes and is not just a consequence of neuritic degeneration. If activation of such imflammatory mechanisms cause neuritic degeneration in plaques as suggested a suppression of IL-6 synthesis could therefore be of therapeutic value. Tenidap belongs to a new class of therapeutic agents. In addition to inhibition of COX, tenidap also inhibits lipoxygenase, superoxide generation and IL-6 production in peripheral blood monocytes. Tenidap was able to inhibit the IL-1 induced synthesis of IL-6 in cultured human astrocytoma cells. Prelimianary data indicate that indomethacin, piroxicam and naproxen are unable to affect IL-6 synthesis. Tenidap inhibition of IL-6 was dose and time dependent and could be demonstrated at both the mRNA and protein level. Tenidap is available for clinical use in the Netherlands and Spain, but awaits approval in other countries [52].

Compounds such as tenidap and others are hindered from going to AD clinical trials for lack of a good animal model of the disease. While transgenic animals are now available which mimic the amyloidosis of AD are now available, it remains to be seen whether the neuroinflammatory components of the pathology will accompany these models. If neuroinflammatory assays could be developed, it would provide a clearer pathway to clinical trials and to the development of inflammatory drugs which operate by a variety of mechanisms.

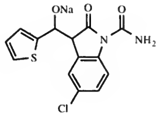
Stroke and Closed Head Injury

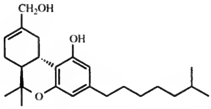
Ischemic stroke and closed head injury are believed to initiate similar pathophysiologic events which involve localized release of excitatory amino acids and subsequent influx of Na^+ and Ca^{++} ions through receptor and voltage gated channels. These events occur on a time scale of minutes to a few hours after the insult. In response to these early homeostatic disruptions, microglia cells are activated and neuroinflammatory cytokines are released. The local expression of cytokines, chemokines and adhesion molecules in the ischemic brain is followed by infiltration of blood-borne neutrophils [53]. These neuroinflammatory events may be responsible for much of the secondary neuronal damage that occurs in the neurodegenerative cascade and disruption of this pathway may represent opportunities to ameliorate neuronal damage. New studies within this area have suggested a number of possible therapeutic mechanisms to explore.

Tumor Necrosis Factor-α

Studies involving interference with TNF-α and its role in neurodegeneration have produced conflicting results. Tools for examining the influence of immunomodulators exist in the form of transgenic mice that are nullizygous for specific ligands, enzymes or receptors. Examination of phenotypes of these animals in varied disease paradigms have provided information about their role in the pathology of certain disease states [54]. Mice that are genetically deficient in TNF receptors (TNFR-KO) have been developed to examine the role of TNF in brain cell injury responses. Damage to neurons caused by focal cerebral ischemia and epileptic seizures was exacerbated in TNFR-KO mice, indicating that TNF may serve a neuroprotective function in this model. Oxidative stress was increased and levels of an anti-oxidant enzyme was reduced in the brain cells of TNFR-KO mice, suggesting that TNF protects neurons by stimulating antioxidant pathways. Injury-induced microglial activation was suppressed in TNFR-KO mice suggesting that the increased damage to neurons was not mediated by microglial toxins [55].

In other studies, inhibition of TNF-α activity has shown an association with cerebroprotection after a rat closed head injury (CHI) model [56]. The TNF-α mRNA and IL-1β mRNA appeared in high amounts within one hour of CHI and remained high as late as twelve hours after the insult. HU-211 **8**, a synthetic cannabinoid, was examined for its effects on TNF-α bioactivity,


Tenidap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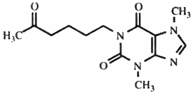
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protein level, and mRNA transcription in the brain after CHI. Treatment of **8** inhibited the surge in rat brain TNF-α bioactivity and TNF-α protein at four hours, but no inhibitory effect on the transcription of.TNF-α mRNA was observed, suggesting that **8** inhibits TNF-α translation or post-translational modifications [57]. Inhibition of TNF-α by **8**, pentoxyfilline **9** and TNF-binding protein correlates with improved neurological outcome after CHI. This observation was extended showing the protective effect of these three different TNF-α inhibitors specifically in BBB integrity, edema formation and on hippocampal neuronal cell survival [57]. Since **8** and **9** have other biological activity in addition to their ability to inhibit TNF-α, it is difficult to definitely state that the protective effects seen in this model are a result of a TNF-α mechanism. Additional agents with more specificity need to be examined in CHI and stroke paradigms to fully elucidate the role TNF-α is playing in acute neurodegenerative pathology.

Interleukin-1

While data suggesting a neuroprotective mechanism of action for TNF-α inhibition is controversal, much stronger evidence indicates a supporting role for IL-1β in stroke pathology. Interleukin-converting enzyme (ICE or caspase 1) is a cysteine protease that is responsible for the cleavage of the proform of IL-1β to the mature cytokine. The enzyme and its inhibitors have been recently reviewed [58,59]. When transgenic ICE knockout mice were subjected to middle cerebral artery occlusion (MCAO), a model for stroke, a significant reduction in ischemic damage was noted compared to wild type [60]. Additionally, IL-1 receptor antagonist administration inhibited ischemic and excitotoxic neuronal damage in a rat focal ischemia model [61,62] and overexpression of IL-1 receptor antagonist reduced ischemic brain injury in mouse brain [63]. These data when taken together make a strong case for the involvement of IL-1β in stroke neurodegeneration. Recently,

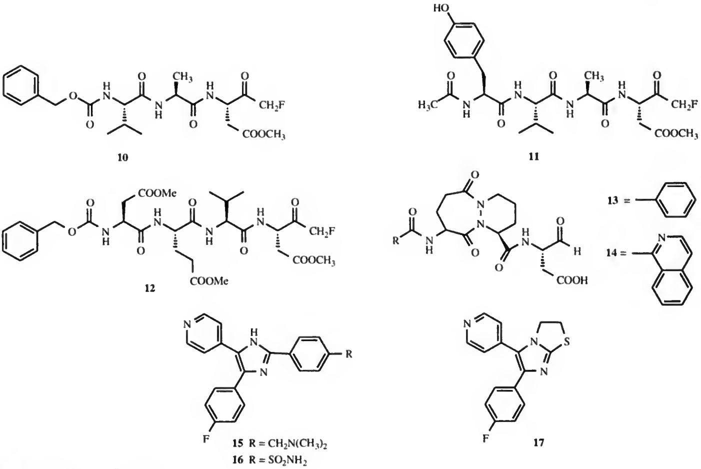
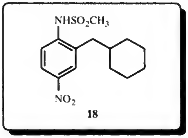


Fig. (5). Inhibitors of IL-1β synthesis.

prodrug peptide inhibitors were injected intracerebroventicularly (icv) in transient and permanent MCAO models [64,65]. In one of the studies, a nonselective peptide inhibitor of the ICE family **10** (at 80 or 240 ng, icv) reduced infarction volume by 40-50%. A second peptide **11** (400 ng, icv) specific for ICE-like caspases decreased infarction volume by 64% (400 ng). Compound **10** reduced toxicity by AMPA and to a lesser extent NMDA suggesting the importance of the ICE family members to glutamate neurotoxicity. Brain swelling was also reduced significantly by administration of **10**. A third peptide **12**, is a more selective inhibitor of another ICE-homolog, CPP32 (caspase-3). CPP-32 is thought to be involved with neuronal apoptosis and not with modulation of inflammation. While **12** (240 ng) reproducibly reduced both infarct size and behavioral deficits, it was weaker than either **10** or **11** and did not block edema formation suggesting a mechanistic difference between inhibitors of CPP-32 and ICE [64]. While there is selectivity between peptides for their respective enzymes, the selectivity is not complete. This is an important point when ascribing a protective effect based on a neuroinflammatory versus apoptotic mechanism. Both mechanisms may contribute to the observed neuroprotection activity and truly selective agents must be developed before the mechanism of action can be unambiguously assigned. This area of research is in need of selective, bioavailable inhibitors that are capable of crossing the BBB. While such agents have not been tested in stroke models, nonpeptide pyridazinodiazepine analogs **13** and **14** are potent ICE inhibitors (Ki = 1 nM) with in vivo activity in inflammation models [66,67]. These compounds could be administered systemically and would represent excellent candidates for testing in stroke paradigms. Smith Kline Beecham has also described several series of pyridylimidazoles which are functional inhibitors of IL-1 biosynthesis. While the precise mechanism of action is unknown, inhibition is believed to be at the translational level. These compounds **15-17** were shown to inhibit IL-1β in LPS-stimulated human monocytes with IC_{50}s of approximately 1 μM. and are interesting candidates for testing in CNS models [68].

Cyclooxygenase

Previous studies have reported the effect on nonselective COX inhibitors (i. e. indomethacin and ibuprophen) on cerebral ischemic damage. The results of these studies have been contradictory. Conflicting observations may be confounded by the effects on these compounds on COX-1 inhibition, an enzyme that is involved in normal cellular function. Further, indomethacin, one of the agents studied most extensively has pronounced effects on cerebral blood flow and vascular reactivity [69]. There is evidence that COX-2 is implicated in the mechanisms of delayed neuronal death at the infarct border, suggesting new neuroprotective strategies targeted at the progression of ischemic brain damage. COX-2 mRNA was upregulated in the ischemic hemisphere, but not contralaterally beginning six hours after ischemia. The upregulation reached a maximum at twelve hours at which time a five fold induction of message was observed. The time course of COX-2 expression closely follows the temporal profile of inflammatory genes, including genes encoding for cytokines, adhesion molecules and inducible nitric oxide synthase (iNOS). Twenty-four hours after ischemia, the concentration of prostaglandin E_2 was elevated in the injured brain by 292 ±57%. COX-2 immunoreactivity was observed in neurons at the medial edge of the ischemic area. Administration of the COX-2 inhibitor NS-398 **18** (20 mg/kg; 2-3 times /day for 3 days) attenuated the elevation in prostaglandin E_2 in the post ischemic brain and reduced the volume of the infarct size by 29 ±6% [69]. Although the factors responsible for decreased cytotoxicity have not heen clearly defined, it is likely that one of the mechanisms is related to the production of reactive oxygen species (ROS). Apoptosis may represent another cytotoxic mechanism since the COX-2 reaction product PGE_2 induces apoptosis in thymocytes [69].



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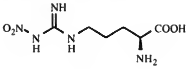
Nitric Oxide Synthase

Within the CNS under normal conditions, nitric oxide (NO) appears to he an important physiological signaling molecule. Its ability to increase cyclic GMP concentrations suggests that NO is implicated in the regulation of important metabolic pathways. Under pathological conditions, NO synthesis may be excessive and become neurotoxic. This area and its implications for neurodegenerative diesases has been recently reviewed [70-72]. A combination of mechanistic and molecular biology has revealed that the active form of NOS is a dimer containing flavin, flavin adenine mononucleotide (FMN), flavin adenine dinucleotide (FAD), heme, reduced nicotinamide adenine dinucleotide phospate (NADPH) and tetrahydrobiopterin [73]. Three major isoforms of the enzyme include the endothelial cell enzyme (eNOS), which is involved in the regulation of smooth muscle relaxation and blood pressure, the neuronal form (nNOS), important to brain development and memory, and an inducible form (iNOS) produced by activated macrophage cells during an immune response. Both eNOS and nNOS are constitutive and calmodulin-dependent while i-NOS is calmodulin-independent [74].

Neurons produce NO mainly by a calcium-dependent activation of nNOS which is expressed constitutively in these cells, whereas glial cells have the ability to synthesize NO in a calcium-independent manner that requires induction of iNOS. The third isoform of NOS, eNOS has been described in the CNS, but is associated with brain vasculature. Activation of nNOS forms part of the cascade pathway triggered by excitatory amino acid release, however, induction of iNOS in glial cells is usually associated with pathological conditions [70,71]. The free radical NO is considered to be a major mediator of immune function in host defense systems. There is evidence that the accumulation of NO mediates damage in inflammed tissue [75,76]. The excessive production of NO by iNOS elicits many deleterious effects which include pain, edema, hyperemia and inflammation. Additionally, this overproduction of NO could be produced through a number of pathways and could result in the activation of COX2 and/or production of free radicals and peroxynitrate [77]. Selective inhibitiors of either nNOS or iNOS may be beneficial in the neuronal degeneration following stroke.



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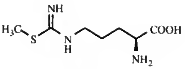

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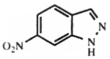

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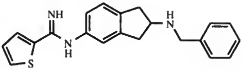

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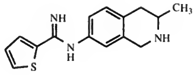

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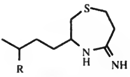

24 R = H

25 R = CH_3

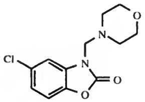

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Fig. (6). Inhibitors of nitric oxide synthase.

To date there are well over a hundred NOS inhibitors in the literature as described in a recent review [73]. Many of these agents are amino acid derivatives which inhibit NOS by competing with the natural substrate arginine. N^{ω}-nitro-L-arginine **19** (2 x 1 mg, ip) only partially inhibited the activity of brain NOS, but it significantly ameliorated early ischemic neuronal damage in a rat model of MCAO [78]. The same group demonstrated a dose-dependent reduction of infarct size after administration of δ-(S-methylisothioureido)-l-norvaline **20** (0.1, 0.3 and 1 mg/kg, ip) in MCAO. Compound **20** is reported to inhibit n-NOS with an IC_{50} value of 5.7 nM [79].

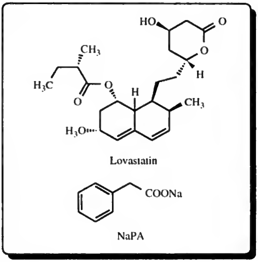
A series of nitroindazoles were examined for NOS inhibition in brain.

7-Nitroindazole (7-NI) **21** was the most potent of these nitric oxide synthase inhibitors with an IC_{50} of 0.9 ± 0.1 μM in rat cerebellum. NOS activity was dependent on exogenous $CaCl_2$ suggesting inhibition of n-NOS [80]. When **21** was dosed (25 mg/kg, ip) after focal ischemia, it significantly reduced infarct volume [81].

Astra has claimed two series of compounds with nNOS activity [82,83]. Compounds **22** and **23** were identified as having rat nNOS inhibitory IC_{50}s of < 10 μM. Patents have claimed that as a result of their selective nNOS activity, these agents should be useful for treatment of hypoxia, stroke and amyotrophic lateral sclerosis [82,83]. Two problems may hinder future clinical exploitation of nNOS inhibitors for CNS indications 1) an incomplete understanding of nNOS-derived NO in CNS function and 2) a range of unwanted side effects which includes impairment of memory acquisition, disordered stomach motility and impotence [73].

Scientists at Merck have claimed several analogs including **24** and **25** which possess submicromolar potency for iNOS inhibition. These compounds are >50 times more selective for iNOS versus eNOS [84]. Another series of benzoxazolones were synthesized and examined as inhibitors of NOS enzymes. The morpholino derivative **26** displayed modest activity and some selectivity for iNOS (iNOS IC_{50} = 0.8 μM; eNOS IC_{50} = 14.7 μM; nNOS IC_{50} = 5.6 μM) [85]. While compounds that inhibit iNOS are most likely to effect stroke pathophysiology via a neuroinflammatory mechanism, selective agents with good brain penetration are required to test this postulate. A recent study, however, has suggested that iNOS induction during and after MCAO occlusion may not be critical for the development of primary infarct damage [86].

In a compelling study by Pahan and coworkers, lovastatin and the sodium salt of phenylacetic acid (NaPA) were observed to inhibit the LPS-induction of iNOS as well as the expression of TNF-α , IL-1β, and IL-6 in rat primary astrocytes, microglia and macrophages [14]. Lovastatin is a potent inhibitor of hydroxymethylglutaryl-CoA reductase (HMG-CoA reductase) and NaPA is an inhibitor of mevalonate pyrophosphate decarboxylase. Both are known to reduce the level of cellular isoprenoids. Inhibition of LPS-mediated iNOS expression by lovastatin and NaPA in astrocytes is suggested to be mediated via NF-κB activation [14].



Lovastatin

NaPA

Immunosupression

FK-506 is a potent neuroprotective agent in an in vivo model of focal cerebral ischemia when administered up to 60 minutes post-occlusion. The minimum effective neuroprotective dose is comparable with the immunosuppressant dose in humans, suggesting that FK506 may have utility for the treatment of stroke. Rapamycin pretreatment blocked the effect of FK506 suggesting that immunophilin binding is essential for neuroprotection. Rapamycin and FK506 compete for a common binding site and suggest that the protein phosphatase calcineurin is implicated in the neuroprotective effect [87].

Multiple Sclerosis

Multiple sclerosis (MS) is a demyelinating disease of the CNS afflicting approximately 250,000 individuals in the U. S [88]. The development of this demyelination is the result of a complex chain of reactions that involves the activation of T-cells and macrophages and the release of numerous cytokines and inflammatory mediators which ultimately leads to CNS damage [89]. A number of immunomodulating therapies have been explored in the clinic and the results of these studies have been reviewed recently [88]. At present, IFN-β–1b (Betaseron) is the only therapeutic agent to convincingly reduce relapse rate and retard disability in the clinic [90]. Other treatments such as corticosteroids, azathioprine, cladribin, total lymphoid irradiation, methotrexate, cyclophosphamide, cyclosporine and mitoxanthrone have been explored with varying degrees of success [88].

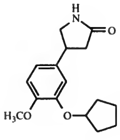
Rodent experimental allergic encephalomyelitis (EAE) has been used as a model to study events associated with MS. This disease model shares features similar to MS in man and is induced by immunization with nervous system-specific myelin basic protein (MBP) in combination with appropriate immunological adjuvants or by adoptive transfer of CNS myelin antigen-specific CD4$^+$ T-cells [91]. Unlike resting lymphocytes, in MS and EAE, activated T-cells express surface adhesion molecules that allow them to cross the intact BBB and invade brain tissue just as they invade other tissues [92,93]. The large number of activated T-cells in the periphery that enter brain tissue can interact with resident microglia and enhance the production of cytokines. Further recruitment of additional cells, notably macrophages, other T-cells and B-cells, can result in the amplification of the initial immune response [93]. Cytokines, specifically IL-1 [94], and TNF-α [95], are induced in microglia in EAE brains. IL-6 in turn is upregulated and may be a major cellular element exacerbating the disease. Microglia and astroglia appear to be a major source of the enzyme NO-synthase and its product nitric oxide [96]. Microglia were identified as the cell type up-regulating such transcription factors as NF-κB in EAE after sciatic nerve transection. These immunocellular events develop a complex series of interactions which are capable of self-propagation and neurodegenerative consequences [97,98]. It may be that molecular mechanisms of microglial activation and differentiation in MS are not as stereotypic as once believed. More insight into these mechanisms would permit much more specific therapeutic strategies in neurodegenerative diseases than presently available [1].

TAK-603 (50 mg/kg/day, po) attentuated the clinical score in EAE rats during the entire experiment. While the specific mechanism of action for the drug is unknown, TAK-603 was also shown to significantly reduce the number of cells responding to MBP. Data suggests that TAK-603 acts during the inductive phase of the immune reaction to reduce the disease-causing or pathogenic antigen-reactive T-cell number [99].

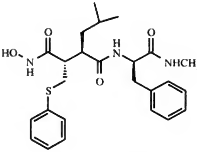
Cytokine and NO Suppression

A number of proinflammatory cytokines such as TNF-α, lymphotoxin (TNF-β), IL-1, IL-6 and IFN-γ have been identified in the brains of MS patients with active lesions [100]. TNF-α has received particular attention because of its pleiotropic effects and its ability to alter the integrity of the BBB. TNF-α exists as a membrane bound precursor form that is processed to yield a 17 kDa secreted form. Studies designed to cast light on its involvement in the development of EAE have produced conflicting results. A number of TNF-α transgenic mice have been studied. In one model, inactivation of the TNF gene converts EAE resistant mice to a high state of susceptibility. Furthermore, treatment with TNF dramatically reduces disease severity in both TNF$^{-/-}$ mice and in other TNF$^{-/+}$ mice highly suspectible to the disease. This study suggests that TNF is not essential for the induction and expression of inflammatory and demyelinating lesions, but it may be beneficial to the diseased animal by limiting the extent and duration of severe CNS pathology [101]. In other studies, TNF-α blocking agents have demonstrated that this cytokine is essential for the development of EAE [102]. Rolipram (Fig. **7**) is an antidepressant in humans, but has recently been reported to have antiinflammatory properties as well. Rolipram increases the intracellular availability of c-AMP levels by selective inhibition of phosphodiesterase type IV. These inhibitors block degradation of c-AMP to AMP resulting in downregulation of the expression of the TNF-α gene. Rolipram reduced hydrogen-peroxide-induced endothelial cell monolayer permeability when combined with PGE$_1$ and inhibited TNF production in LPS-stimulated human mononuclear blood cells. Additionally, it suppressed TNF production by autoreactive rat and human T-cells and most importantly ameliorated the symptoms in clinically manifest disease [103]. A second study suggested that rolipram inhibited the migration of leukocytes into the CNS and reduced T-cell proliferation and macrophage activity. This study, however, demonstrated no benefit when rolipram was administered (2 x 3 mg/kg/day) starting on the day of immunization with MBP [104]. Pentoxyifylline **9**, another inhibitor of PDE type IV, was tested in an EAE model in Lewis rats and almost completely abrogated the disease during the induction phase. Lack of clinical symptoms in pentoxyifylline-treated animals correlated with a marked suppression of MBP-specific T-cell reactivity in vitro [105,106]. TNF-α exists as a membrane bound precursor form that is processed by TNF-α converting enzyme (TACE) to yield a 17 kDa secreted form. TACE is a metalloprotease and represents a novel target in the treatment of a variety of TNF-α associated diseases [107]. GW9471 is a hydroxamic acid that can inhibit TACE. GW9471 showed a dose-dependent reduction of TNF-α from LPS-stimulated THP cells, but no testing in EAE paradigms have been reported [107].

Cytokines such as IL-1β are thought to contribute to the inflammatory response observed with autoimmune diseases like MS. Interleukin-1 receptor antagonist (IL-1ra) was tested in a rat model of EAE and significantly reduced the severity of paralysis and weight loss and shortened the duration of the disease [91]. In



Rolipram

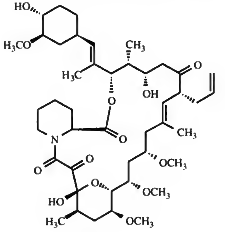


GW9471

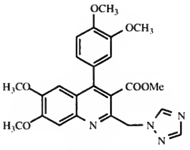
Fig. (7). Inhibitors of TNF-α production.

a second experiment, soluble mouse IL-1 receptor which binds both IL-1α and IL-1β was given for 13 consecutive days. The soluble receptor significantly delayed the onset of EAE, reduced the severity of the paralysis and weight loss, and shortened the duration of the disease. These data suggest that IL-1 may initiate or promote inflammation within the CNS[108].

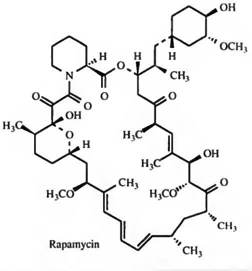
An increase in the mRNA of cytokines known to induce iNOS (i.e. IL-1α, IL-1β, IL-6, TNF-α and IFN-γ) was observed to correspond to the severity of the disease during the course of EAE experiments. A rapid decline in the gene expression of these immunomodulators was then observed in the spinal cord during the recovery phase of EAE [75]. Aminoguanidine (AG), a nucleophilic hydrazine compound, was reported to be a potent and selective inhibitor of iNOS. AG significantly reduced EAE disease incidence, severity, and duration with a concomitant decline in cellular infiltrates and demyelination. AG (8 mg/mouse/day, ip) completely suppressed clinical expression of EAE in 17 of 27 treated mice when administered simultaneously with disease induction [89]. In a post-mortem study using Northern blot analysis and reverse transcriptase in situ PCR, iNOS was present in brain tissues from all seven MS patients, but was absent in equivalent tissues from normal controls [109].



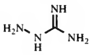
FK-506



TAK-603



Rapamycin



AG

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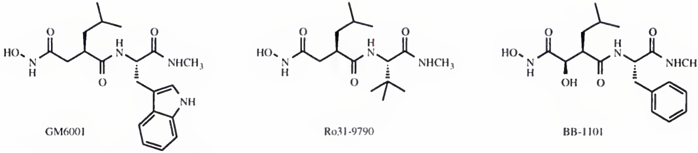

GM6001 Ro31-9790 BB-1101

Fig. (8). Inhibitors of matrix metalloproteases.

This evidence when examined together makes a strong argument that a reduction of cytokine and NO levels would provide a rationale mechanism to ameliorate the symptoms of MS. Small molecule inhibitors of these immunomodulatory mechanisms await development and testing.

Matrix Metalloproteinase Inhibition

The matrix metalloproteinases (MMPs) are a family of enzymes which contain a zinc atom in the active site of the catalytic domain and include the collagenases, gelatinases, and stromelysins. MMPs have the potential for massive tissue destruction and thus are tightly regulated [110]. MMP inhibitors may contribute to ameliorative effects seen in EAE based on two mechanisms: 1) by blocking the extravasation to the CNS and effector properties of lymphocytes and macrophages in the initial stage of inflammation, and 2) by limiting the myelin loss in the established lesions [111]. Gelatinase A (MMP-2) and B (MMP-9) are capable of enzymatic digestion of the subendothelial basement membrane constituents. IL-2 induces gelatinase secretion and enhances gelatinase-dependent migration across an artificial basement-membrane-like layer [112]. Two broad spectrum MMP inhibitors GM6001 and Ro31-9790 have been shown to reduce disease in acute EAE models in the rat [110,111]. The beneficial effect of Ro-31-9790 was greatest in animals with moderate clinical signs declining in those with more severe symptoms [111]. BB-1101, another broad spectrum MMP inhibitor, is a potent inhibitor of TNF-α production in vivo and inhibits both the inflammation and demyelination observed in EAE. [110].

As mentioned above, IFN-1β has substantial clinical benefit in MS, yet the mechanism of action remains largely unknown. Pretreatment of T-cells with INF-1β for 48 hours decreased IL-2-induced gelatinase production. The inhibitory effect mediated by treatment with INF-1β was dose-dependent and the raises the possibility that its beneficial effects in MS may result from direct interference with the capacity of activated T-cells to traverse the basement membrane and migrate to the CNS [112].

Conclusion

Interference with neuroinflammatory processes may offer opportunities for ameliorating the effects of many devastating neurodegenerative diseases. Data supporting the role of neuroinflammation in such major diseases as AD, stroke, traumatic brain injury and MS have been discussed in this review, but there are many other CNS debilitating diseases such as Parkinson's disease, myasthena gravis, amyotrophic lateral sclerosis and HIV-dementia where inflammation is also a damaging component. There are presently a plethora of molecular mechanisms to consider, but many problems still remain if experimentalists are to produce effective drugs for neuroinflammatory diseases. The literature describing many molecular targets such as the enzymes ICE, iNOS, and TACE has only recently produced active, bioavailable compounds that offer opportunities for in vivo testing. There are also disease states such as AD where no appropriate animal models exist and this retards compound development. Additionally, activation of distinct receptor mechanisms and potential-initiation of responses may be specific for the type of injury or disease. Thus, it may be that microglial activation and differentiation are not as stereotypic as once thought. More insight into these molecular mechanisms would permit more specific therapeutic strategies for halting the progression of neurodegeneration. Understanding these processes represents a formidible challenge for future neuroscientists.

Abbreviations

AD = Alzheimer's disease
APP = Amyloid precursor protein
BBB = Blood brain barrier
CNS = Central nervous system
CHI = Closed head injury model
COX = Cyclooxygenase
EAE = Experimental allergic encephalomyelitis
eNOS = Endothelial nitric oxide synthase
FAD = Flavin adenine dinucleotide
FMN = Flavin adenine mononucleotide
HMG-CoA reductase = Hydroxymethylglutaryl-CoA reductase
IL-1α = Interleukin-1α
IL-1β = Interleukin-1β
IL-2 = Interleukin-2
IL-3 = Interleukin-3
IL-6 = Interleukin-6
IgG = Immunoglobin G
iNOS = Inducible nitric oxide synthase

INF-β = Interferon-β
INF-γ = Interferon-γ
ICE = Interleukin converting enzyme
LPS = Lipopolysaccarides
MBP = Myelin basic protein
MCAO = Middle cerebral artery occlusion
MMP = Matrix metalloprotease
MS = Multiple sclerosis
NADPH = Reduced nicotinamide adenine dinucleotide phosphate
NaPA = Sodium salt of phenyl acetic acid
NMDA = N-Methyl-D-aspartate
nNOS = Neuronal nitric oxide synthase
NO = Nitric oxide
NOS = Nitric oxide synthase
NSAID = Nonsteroidal antiinflammatory drug
PG = Prostaglandin
ROS = Reactive oxygen species
TNF-α = Tumor necrosis factor-α
TNF-β = Lymphotoxin
TNFR-KO = TNF receptor knockout mice

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Central Control of Feeding Behavior by Neuropeptide Y

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Abstract: Obesity is a serious health problem in the Western societies, therefore its treatment has become the subject of intense interest in the scientific community. A significant number of recent publications enlist different central and peripheral factors which play important roles in the regulation of food intake, body weight and energy expenditure. Neuropeptide Y, a 36 amino acid peptide, which is quite abundant in the brain, seems to be one of the more important players in these regulations. Recently five NPY receptors have been cloned and pharmacological evidence strongly supports the existence of a sixth receptor. There are many contradictory findings regarding which NPY receptor mediates the effect of NPY on food intake. This article will review the effects of NPY on the regulation of food intake and energy expenditure and will discuss the pharmacological and molecular evidence as to which NPY receptor(s) mediate this effect. The review will also summarize the progress which has been made in the design of novel NPY-ergic ligands, especially NPY receptor antagonists, for potential use in the treatment of obesity.

Introduction

There have been many reviews in recent years addressing the potential roles of peptides in the regulation of energy homeostasis. There is no doubt that NPY is one of the key players in this process. This review will focus on summarizing recent drug discovery approaches for the treatment of obesity through effects on NPY-ergic mediated responses.

Obesity is a major health problem in the Western Societies affecting a third of Americans [1,2]. Even mild obesity enhances the risk of premature death, hypertension, diabetes mellitus, hyperlipidaemia, atherosclerosis, coronary heart disease, arthritis, sleep apnea and certain types of cancer [3,4]. There is a strong association of obesity and non-insulin dependent diabetes mellitus (NIDDM), and more than 80% of NIDDM patients are obese [5].

The etiology of human obesity is still unknown. The early view of obesity was that it was the result of compulsive overeating. Today it is clear that there are multiple causes for the development of obesity of physiological, genetic and environmental nature [6]. It has been shown that significant weight loss reduces the risk of the aforementioned diseases [7,8]. Therefore one of the major challenges of the pharmaceutical industry along with the healthcare system is to achieve sufficient, long-term weight loss to improve the overall health of the population. The successful approach may include behavioral modification along with chronic, safe, and non-addictive drug therapy.

For the successful development of an effective pharmacotherapy, an understanding of the pathophysiology of obesity is essential. Extensive research has provided evidence that both peripheral and central factors are involved in energy homeostasis. This review will focus on the involvement of the central nervous system. Amongst the many neurotransmitters synthesized and released in the brain, NPY has perhaps received the most attention since the late 1960's. The NPY story started with the discovery of a relative to NPY, pancreatic polypeptide (PP) [9,10]. Soon after this discovery, PP was also described in many species. Tatemoto and Mutt [11] reported in 1980 a PP-like peptide isolated from porcine brain and gut. This peptide was named pancreatic polypeptide Y (PYY) since both the N- and C-terminal amino acids were found to be tyrosine (Y). It was subsequently found that the "brain" PP is different from the "gut" PYY, which led to the discovery of the 36 C-aminated amino acid NPY [12,13]. To date, NPY is the most abundant neuropeptide found in the brain, particularly in the hypothalamus [14]. NPY is synthesized primarily in the arcuate nucleus of the hypothalamus which has nerve fibers projecting to the paraventricular nucleus (PVN), to the dorsomedial hypothalamus and to the lateral hypothalamus [15]. The PVN also receives NPY-ergic innervation from the brain stem (medulla), where NPY is co-localized with norepinephrine [16-18]. Other hypothalamic regions with high NPY levels are the periventricular area close to the third ventricle, the medial preoptic area and the suprachiasmatic and supraoptic nuclei [19,20].

There have been at least 40 members of the pancreatic polypeptide family identified to date. The sequences of NPY are known for many species from lamprey to mammals and is one of the most highly conserved neuropeptides known. Out of 36 positions, 22 of the amino acids are identical in all of the investigated species, including fish, chicken, pig, cow, sheep, rat, rabbit, guinea pig and man [21]. In comparison, PYY has only 15 and PP only 7 well conserved amino acids [22]. Cloning of the NPY gene also demonstrates conservation of its intron and exon structure [23-26]. Such remarkable conservation of NPY's structure would imply an important and ubiquitous role for this neuropeptide throughout the animal kingdom.

Many scientific communications and reviews describe the many physiological roles of NPY. These include cardiovascular effects [27,28], effects on release of other neurotransmitters

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[29], its role in affective disorders [30,31] and in reproductive function [32]. Recent reviews have also described the physiological roles of NPY receptors in obesity [33-36]. This review will focus on the advances of pharmacotherapy for the treatment of obesity targeting neuropeptide Y receptors.

The Effect of Neuropeptide Y on Food Intake

NPY is the most potent stimulator of food intake known [37-40]. The orexigenic effect of NPY has been demonstrated in a wide variety of animal species including rats, mice, chicks, hamsters, ground squirrels, pigs, snakes, sheep, white-crowned sparrows, and rhesus macaque monkeys [41-48]. NPY demonstrated the highest efficacy on food intake when administered into the PVN of the hypothalamus, but it was also effective after intracerebroventricular (i.c.v.) administration [49, 50].

The behavioral effect of NPY on feeding has many different aspects. Centrally administered NPY decreases the latency of feeding initiation, and increases the amount of food consumed without altering the frequency of feeding [49,51,52]. This suggests that NPY reduces the sensation of satiety. Chronic central administration of NPY has found that its orexigenic effect persists during treatment without the development of tolerance [53]. Studies also suggest that NPY induces a preference for carbohydrate consumption over that of fat or protein [54]. The effect of NPY on food intake does not appear to be tied to the circadian rhythms of the animals [55, 56].

There are three lines of evidence that NPY is a major regulator of food intake. First, chronic i.c.v. administration of an antisense phosphorothioate oligonucleotide complementary to the rat mRNA for NPY significantly reduced cumulative food intake, meal size and meal duration compared to missense treated control rats [57]. The antisense treated rats weighed less at the end of the study compared to the missense treated controls and this weight reduction was reversible upon cessation of treatment. Second, infusion of anti-NPY antiserum into the third ventricle or above the PVN reduces the feeding response upon fasting [58, 59], and third, stimulation of endogenous KCl-induced NPY release in freely moving rats increases food intake [60].

Obviously we would not like to leave the reader with the feeling that NPY is the only neuropeptide or neurotransmitter affecting food intake. However, it should be emphasized that the NPY-ergic system is one of the major regulatory pathways in this extremely complex and vital function. There are at least 40 different neurotransmitters identified in the rat hypothalamus most of which have been shown to have an effect on food intake. In this review we will note some of the key interactions of the NPY-ergic system with insulin, glucocorticoids and leptin, which are other major physiological mediators of the energy homeostasis.

A detailed investigation of the molecular mechanism of energy expenditure and metabolism started with the discovery of insulin which shed more light on the control of glucose metabolism and energy storage. It has been demonstrated that both central and peripheral insulin infusion reduces the fasting-induced stimulation of NPY levels in the hypothalamus [61, 62] due to reduced NPY gene expression and peptide release [63]. Conversely other found that insulin increase NPY levels. After a 3-day subcutaneous insulin infusion (co-infusion with glucose to prevent hypoglycemia) NPY levels were found to be higher in the PVN than in control animals [64]. However, this result could possibly be explained by an insulin-induced inhibition of NPY release in the PVN which causes local intracellular NPY accumulation. Generally, it appears that centrally administered NPY enhances insulin secretion [65, 66] while peripheral NPY released from sympathetic neurons inhibits it [67]. Although it is clear that both NPY and insulin have mutual effects on the synthesis and release of the other peptide, the modulatory mechanisms require further clarification. It is important to note that there is a high correlation between diabetes and obesity [3,33,35]. Adequate weight loss in diabetic patients enhances the effects of hypoglycemic agents. Conversely, obese individuals are at significantly greater risk to develop non-insulin dependent diabetes.

In addition to insulin, many studies suggest that glucocorticoids, such as dexamethasone, enhance expression of NPY in PC12 cells and in hypothalamic cultures, as well as increase NPY release and food intake [68,69]. Tempel and Leibowitz [70] showed that stimulation of carbohydrate intake by centrally administered NPY was abolished after adrenalectomy, however it could be restored upon corticosteroid administration. It was shown that the permissive effects of glucocorticoids on the NPY-induced feeding response occurs through the type II glucocorticoid receptor in the PVN of the hypothalamus. Furthermore, adrenalectomy prevents the development of obesity by chronic central administration of NPY which suggests that intact adrenal glands and normal corticosterone levels are necessary for the central effects of NPY [71]. Conversely, NPY injected into the PVN induces corticotropin secretion presumably by stimulating neurons which release corticotropin releasing factor (CRF) [72]. This speculation is supported by the finding that NPY-immunoreactive neurons have been found to form synapses with CRF-containing neurons in the PVN [73]. NPY stimulates feeding and the release of corticotropin with very similar potency. This finding indicates that NPY also regulates the hypothalamic-pituitary-adrenal axis integrating feeding and adrenocortical functions. Unlike stimulation of insulin secretion which is projected to negatively regulate NPY expression and synthesis, there is a positive feedback regulation between NPY and glucocorticoids. CRF has been shown to have anorectic effects on its own [74] and it may inhibit NPY secretion [75] to provide a balance for energy expenditure.

The recent discovery of lenptin, a protein expressed selectively in adipose tissue, has shed new light on an understanding of body weight regulation [76]. Administration of leptin to normal mice reduces food intake and body weight [77-79]. It appears that leptin's major role is to signal the fat content of the body to the brain. Several recent studies have suggested that leptin binding to hypothalamic membranes reduces hypothalamic NPY release [80,81]. In lean animals there is a loop system which connects the peripheral adipose pool with the central regulatory systems in the hypothalamus. Increasing NPY levels enhance corticosterone and insulin levels in the periphery, which enhance glucose mobilization, food intake and storage into fat. The increased levels of insulin will ultimately enhance lipase activity in adipose tissue resulting in increased plasma leptin levels. These in turn will reduce NPY levels in the brain, thus closing the regulatory loop. In obese animals (Zucker fatty rats, *ob/ob* mice) this loop system is dysfunctional since they do not have sufficient amounts of leptin required to reduce NPY levels.

Besides the aforementioned neurotransmitters and hormones there are further connections between NPY and adipose tissue. NPY injected into the PVN dramatically alters energy balance not only by causing overeating but by reducing energy expenditure via inhibition of the sympathetic stimulation of brown adipose tissue [65]. Centrally administered NPY also augments white adipose tissue lipoprotein lipase activity [82]. NPY thus induces an autonomic imbalance, which causes increased insulin levels, reduced energy use and increased fat storage.

Cold exposure also has an effect on NPY levels. It has been shown that 18 hrs of cold exposure increases NPY concentration in the PVN and ventromedial nuclei (VMN) without changing the message level of NPY. These findings suggest that increased brown fat thermogenic capacity induced by cold may be mediated by decreased NPY release in the PVN, therefore resulting in its accumulation at this site [83]. In addition to cold exposure, other situations of negative energy balance such as starvation and lactation have been shown to be accompanied by elevated NPY level in the hypothalamus [84, 85].

The elucidation of numerous biological factors on the expression, synthesis and release of NPY is far from complete. It does, however, emphasize the complexity of the regulation of the energy homeostasis. In summary the literature provides evidence that:

(1) NPY is one of the most important players in the regulation of body weight and energy expenditure.

(2) Regulatory systems which provide satiation reduce NPY levels in the brain.

(3) Appetite stimulating systems increase NPY levels in the brain.

(4) Enhanced level of NPY may contribute to the etiology of obesity.

An effective treatment of obesity therefor can be achieved if the NPY levels in the brain are reduced or if the action of NPY on the relevant NPY receptors is inhibited.

To date, five NPY receptors have been cloned and pharmacological evidence support the existence of others. In subsequent sections, we will summarize the cloning and pharmacological characterization of these receptors and describe both molecular and pharmacological evidence-as to which of these receptors transduce the effect of NPY on food intake. For the purpose of clarity, recently accepted IUPHAR nomenclature of the NPY receptors will be used herein.

Molecular Biology of NPY Receptors

Y_1 Receptor

Molecular cloning of the first NPY receptor subtype, the human Y_1 receptor, was aided by noting similarities in the expression pattern of FC5, a rat orphan G protein-coupled receptor [86], as demonstrated by *in situ* hybridization, to that of Y_1 receptor protein, as shown by autoradiography. Rat FC5 coding sequences were used as a probe to screen and isolate Y_1 receptor cDNA from human fetal brain library [87,88]. As expected, the human Y_1 receptor was shown to be a member of the G protein-coupled receptor family. Sequence comparisons have revealed a high degree of Y_1 receptor conservation between human [87,88], rat [86] and mouse [89], with overall identities of ≥ 94%. The human Y_1 gene has been mapped physically to chromosome 4q31.3-32 [90]. The genomic structure of human and mouse Y_1 genes has been reported and shown to be conserved [89-91]. Both human and mouse Y_1 coding sequences are contained in two coding exons, interrupted by an intron positioned shortly after the proposed fifth transmembrane domain. This feature is unique to the Y_1 receptor, as coding sequences of all other cloned Y receptor genes are uninterrupted. In mouse, a splice isoform encoding a truncated Y_1 receptor protein, missing sequences of the seventh transmembrane domain and beyond, has been identified [89]. Expression of the truncated mouse Y_1 isoform is detected in bone marrow cells and at early stage of the embryonic development. NPY appears to bind to this truncated isoform, but does not induce a functional response [89]. Y_1 receptor mRNA is expressed in brain, and several peripheral tissues, including heart, kidney, spleen, skeletal muscle and lung [89]. Regional Y_1 receptor mRNA distribution in rat and human brain sections is similar [86,91,92]. Expression is seen within hippocampus, thalamus, amygdala, cortex and to a lesser extent, in hypothalamic nuclei. It is of note that a good correlation exists between Y_1 receptor mRNA expression and specific Y_1 binding sites in the rat central nervous system [92]. Heterologously expressed human Y_1 receptors couple to inhibition of forskolin-stimulated cAMP accumulation as well as elevation of intracellular Ca^{2+} [88,89,93]. Similar signal transduction pathways have been reported for SK-N-MC cells [94], which are Y_1 expressing human neuroblastoma cells used most commonly to pharmacologically characterize human Y_1 receptors.

Y_2 Receptor

The human Y_2 receptor was cloned using number of different expression cloning techniques [95-98]. Sequence comparisons showed low levels of conservation between human Y_1 and Y_2 receptors, with about 31% overall identity. The Y_2 receptor gene (NPYR2) maps to human chromosome 4q31 [99], which also contains the Y_1 receptor locus [90]. By Northern blot analysis, widespread human Y_2 receptor mRNA expression is seen in a variety of brain regions [95,98]. However, with the sole exception of the small intestine, most peripheral tissues examined display no hybridization. Activation of heterologously expressed human Y_2 receptor leads to inhibition of forskolin-stimulated cAMP accumulation and mobilization of intracellular Ca^{2+} levels [95,96]. Human neuroblastoma cell lines SMS-KAN and SK-N-BE(2) express endogenous Y_2 receptors which have been shown to couple to inhibition of adenylate cyclase [100, 101]. Pharmacologically, the Y_2 receptor has been found both prejunctionally in vas deferens [102] and postjunctionally in cardiomyocytes [103].

Y_3 Receptor

This receptor has not yet been cloned but substantial pharmacological evidence support its existence. This is the only known NPY receptor which does not recognize PYY as a ligand. The Y_3 receptor was first found in the adrenal medulla [104], and later in cardiac membranes [105,106] and brain stem [107]. Bovine cromaffin cells were also shown to have a PYY-insensitive NPY receptor [101], coupled to Ca^{2x+} influx, but not adenylate cyclase. Recent pharmacological characterization of NPY/PYY chimeras and other NPY analogs suggested that the 13th and 14th amino acids of NPY play a critical role in the affinity of the receptor for these peptides [108]. [1-23]NPY-[24-36]PYY and [1-14]NPY-[15-36]PYY are the highest affinity agonists, while the receptor does not recognize [Leu^{31}, Pro^{34}]NPY (see Table 1.).

Y_4 Receptor

Through homology screening based on the Y_1 receptor sequence information, the Y_4 receptor has been cloned from several species including human [109,110], rat [110,111], and mouse [112]. In some of the earlier studies, the Y_4 receptor, which binds PP with high affinity, was designated as PP1. Structurally, the human Y_4 receptor is most related to the human Y_1 and Y_6 receptors, with 42% and 38% overall identity, respectively. For comparison, the structural identity with the human Y_2 and Y_5 receptors are only 33% and 32%, respectively. The amino acid sequence identity between the mouse/rat and human Y_4 receptors is only 76%/75% compared to ≥90% typically observed for other species homologs. This high degree of sequence divergence could reflect the fact that the receptor's preferred ligand, PP, is less conserved between species than is NPY. Furthermore, the pharmacological profile of the Y_4 receptor is considerably different between human and rat; the rat Y_4 receptor binds PYY and NPY with significantly lower affinities than its human orthology [111]. Human Y_4 receptor mRNA expression is found in a number of peripheral tissues including skeletal muscle, lung, gut tissues, liver, kidney, heart, testis, and prostate [109,110,112]. Human Y_4 receptor mRNA expression has also been detected in a number of different brain regions, including hippocampus, thalamus, amygdala and hypothalamus [109,110,112]. Activation of the human Y_4 receptor expressed in heterologous systems results in inhibition of forskolin-stimulated cAMP accumulation and eleuation of intracellular Ca^{2+} levels [109,111].

Y_5 Receptor

In search of the $Y_{feeding}$ receptor, expression cloning was used successfully to isolate a rat hypothalamus cDNA encoding the Y_5 receptor [113,114]. Genomic and cDNA sequences encoding the human and mouse Y_5 receptor, respectively, have also been isolated [114,115]. Sequence comparisons indicate that the human, rat and mouse Y_5 receptors exhibit high degree of conservation, sharing 88-97% overall identity. Y_5 receptors, however, exhibit very low identity to other cloned Y receptor, subtypes, with 30-33% overall identity. Human Y_1 and Y_5 receptor genes exist as overlapping and divergent transcriptional units sharing a common promoter region on chromosome 4q31-32 [116]. This suggests that regulation of Y_5 and Y_1 gene expression may be coordinated. A similar transcriptional organization has also been reported for the mouse Y_5 and Y_1 genes [115]. By Northern blot analysis, rat Y_5 mRNA is reported to be expressed in a number of peripheral tissues including spleen, stomach, kidney, small intestine, adrenal gland and pancreas [114]. Abundant rat Y_5 mRNA expression is also detected in the whole brain and in lower levels in the hypothalamus, hippocampus, cortex and olfactory bulb. By *in situ* hybridization, high levels of rat Y_5 mRNA expression is found in hypothalamic nuclei implicated in feeding, including the lateral hypothalamus and paraventricular nucleus [113]. Heterologously expressed human and rat Y_5 receptors couple to the inhibition of adenylate cyclase [113, 117].

Y_6 Receptor

Homology screening using Y_1 receptor sequences as probes and polymerase chain reaction (PCR) approaches, have led to identification of the Y_6 receptor [118-120]. The Y_6 receptor was designated either Y_5, Y_{2b} or PP2 in early literature reports prior to the publication of the IUPHAR approved nomenclature. The Y_6 gene encodes a functional receptor protein in mouse and rabbit. It is absent in rat and exists as a pseudogene in humans (y_6) as well as in other higher order primates [118-120]. This is indicative of a highly divergent y_6 gene evolution in mammals. Sequence comparisons show that percent identities of the human y_6 receptor to human Y_1, human Y_4 and human Y_2 receptors are 51%, 38 % and 29%, respectively. The human y_6 receptor gene exists as a single copy which maps to human chromosome 5q31 [120]. The reported human y_6 gene and cDNA sequences contain a single base deletion in the coding region. This results in a non-functional truncated y_6 receptor protein lacking sequences beyond the sixth transmembrane domain [119, 120]. The inactive human y_6 receptor gene is highly expressed in heart, skeletal muscle, spleen, prostate and small intestine. By reverse transcriptase-PCR analysis, rabbit y_6 receptor mRNA is detected in the hypothalamus. Peripherally, the receptor is also found in the small intestine, colon and adrenal gland. By *in situ* hybridization, mouse Y_6 mRNA expression is detected in several hypothalamic nuclei, including the ventromedial nucleus [118]. Although the function of mouse Y_6 receptor is currently unknown, its hypothalamic site of expression and Y_1-like pharmacology warrants caution when interpreting results of whole animal feeding studies in mice.

Pharmacology of NPY Receptors

Peptide Ligands

A large body of literature describes receptor binding studies to different NPY receptors using members of the pancreatic polypeptide family as well as their analogs. Although there are discrepancies as to the rank order of potency of the different peptides to NPY receptor subtypes depending on the radiolabeled ligand used, there is a widely accepted consensus regarding the potency and selectivity of some of these peptide ligands. Table 1 summarizes the rank order of potencies of various pancreatic polypeptide ligands to the NPY receptor subtypes.

A considerable amount of effort has been directed towards the identification of new and selective peptidic ligands. The area has been recently reviewed by Balasubramanian [121]. The major

Table 1. Pharmacological Characterization of NPY Receptor Subtypes

Receptor	Ligand Potency	Reference
Y_1	Leu^{31}Pro^{34}-NPY>PYY=NPY>NPY2-36>>NPY 13-36>PP	87
Y_2	PYY>NPY>NPY 13-36>>Pro^{34}-NPY>PP	99
Y_3	NPY=Pro^{34}-NPY>NPY 13-36>NPY 18-36=PP>>>>PYY=Leu^{31}Pro^{34}-NPY	108
Y_4	PP>PYY=NPY= Leu^{31}Pro^{34}-NPY>NPY 2-36	110
Y_5	NPY=PYY=NPY 2-36>NPY 3-36>NPY 13-36	113
y_6	NPY=PYY$\geq$ NPY 2-36= Leu^{31}Pro^{34}-NPY>NPY13-36>PP	118

goal of these studies was to find selective ligands for NPY receptor subtypes in order to complete their pharmacological characterization and more fully elucidate their physiological functions. A series of linear pentapeptides has been synthesized that displayed low micromolar affinities for rat brain NPY receptors. Some of these were antagonists at the Y_1 receptor subtype, as measured by Ca^{2+} flux studies in HEL cells endogenously expressing the Y_1 receptor [94]. The inactive NPY carboxyterminal pentapeptide (ThrArgGlnArgTyr-NH_2) was modified by replacing the threonine unit with an aromatic amino acid and the glutamine unit with leucine. These modifications resulted in a series of pentapeptide ligands with <1 μM affinities [122]. A series of truncated analogs were prepared by removing the central turn region as well as replacing some of the N- and C-terminus helical regions with an 8-aminooctanoic acid residue stabilized by a disulfide bridge [123]. One of the more potent analogs, ([D-Cys^{7}-Aoc$^{8-17}$-Cys^{20}]pNPY), displayed as high affinity to mouse brain receptors as NPY itself. In SK-N-MC cells, which express the Y_1 receptor subtype, it was shown that this truncated NPY analog was an agonist [124].

Replacement of NPY's amino acids with D-Trp resulted in several analogs which had high affinities and high intrinsic activities. Of these, [D-Trp^{32}]NPY had high affinity to rat hypothalamic membranes, but it had no intrinsic activity measured by adenylate cyclase activity in rat hypothalamic membranes. The compound also reduced NPY-induced 1 hr feeding responses in the rat suggesting that [D-Trp^{32}]NPY was a competitive antagonist of NPY [125]. More recent data suggests that [D-Trp^{32}]NPY is a low-affinity ligand at the Y_1 and Y_2 receptors, but it has nanomolar affinity at the Y_5 receptor as an agonist [113]. Balasubramanian *et al.* have also reported a number of Y_1 vs. Y_2 receptor selective peptidic antagonists with nanomolar (36-143 nM) affinities. These peptides were designed based on the C-terminal hexapeptide of NPY [126].

Scientists at Glaxo-Wellcome synthesized several peptides of various sizes which act as moderately selective antagonists at the Y_1 receptor subtype with at or below nanomolar affinities [127]. The most potent of these peptides, 1229U91 has been extensively studied over the past year. The structure of 1229U91 is (IleGluProDprTyrArgLeuArgTyrNH_2)$_2$, where Dpr is 2,3-diaminopropionoic acid, and the dimer connected by two amide bonds between the Dpr and Glu units. 1229U91 was found to have an affinity of 18 pM at the Y_1 receptor. The compound was selective for the Y_1 receptor since it showed little affinity for Y_2 and Y_5 receptors and only moderate affinity (7.2 nM) for the Y_4 receptor [128,129]. Moreover it was found to be an antagonist at the Y_1 receptor, while having intrinsic activity at the Y_4 receptor. 1229U91 also inhibited NPY-induced feeding in the rat, providing pharmacological evidence that the Y_1 receptor is involved in NPY's action in feeding.

Extensive efforts have also been conducted in recent years in the design of selective Y_2 receptor agonists and antagonists. Pharmacological and NMR evidence have revealed that the alpha-helix content of NPY is not the predominant factor for high-affinity Y_2 receptor binding. Instead, the size and the location of the hydrophobic lactam bridge, and the conserved C-terminal tetrapeptide (ArgGluArgTyr) are the critical features necessary for recognition [130]. A number of cyclic peptides have been synthesized which posses selective high affinity binding to the Y_2 receptor subtype. NMR studies of the cyclic peptides suggest that a turn-like structure and the orientation of the C-terminus toward the N-terminus play major roles for high-affinity binding of cyclic dodecapeptides to the Y_2 receptor. Since these peptides are also selective for the Y_2 receptor and do not bind to the Y_1, their folding support the hypothesis that the binding site of NPY at the Y_1 receptor is discontinuous. Using a cyclic template containing two β-turn mimetics for covalent attachment of the carboxy terminal fragments, T4-[NPY(33-36)]$_4$ was found to be a potent and selective antagonist at the Y_2 receptor [131]. To date, no other peptidic ligands for the Y_3, Y_4, Y_5 and Y_6 receptors have been reported.

Non-Peptidic NPY Receptor Antagonists

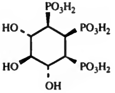
Since 1990 a number of potent, non-peptidic NPY receptor antagonists have been reported in the literature. Most of the antagonists have been identified either through a rational design approach based on mimicking key structural features of either NPY and/or a pharmacophore binding motif, or by serendipitous discovery through screening large chemical compound collections. Many of the early antagonists were first characterized by their ability to modulate NPY-induced effects on vascular tissue. Thus for the most part, little if any anorectic behavior of any of these agents has been reported. Additionally, many of the newer agents are reported only in the patent literature which doesn't necessarily correlate specific biological data with specific antagonists. Never-the-less, a considerable amount of information is available on these antagonists which can potentially provide important information and/or direction for future design and development of second generation NPY antagonists. A number of reviews have appeared since 1992 which summarize much of this earlier material [35,132-136].

Y_1 Receptor Antagonists

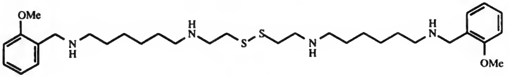
The first reported non-peptidic Y_1 receptor antagonist was D-*myo*-1,2,6-inositol triphosphate (**1**) [137,138]. The compound significantly decreased the NPY-induced contractile response in a guinea pig basilar artery preparation at 1 and 10 µM . Although the antagonism was specific for NPY, Schild plot analysis revealed it to be a non-competitive interaction. Subsequent studies with inositol **1** in HEL and SK-N-MC cells found that it had no effect on [^{125}I]NPY binding [139]. The compound had no effect on NPY-stimulated increase in Ca^{2+} levels in HEL and SK-N-MC cells and did not antagonize forskolin-stimulated cAMP formation in HEL cells. Similarly, [^{125}I]NPY binding in membranes from rat brain and in SK-N-BE(2) cells was unaffected by **1** suggesting the absence of affinity at other NPY receptors [140]. It was concluded that the effect of D-*myo*-1,2,6-inositol triphosphate occurs at a point downstream from the NPY receptor or second messenger systems in these tissues and cell lines. Never-the-less, the compound did demonstrate *in vivo* anorectic activity. When dosed at 20 nmol i.c.v. into Sprague-Dawley rats, D-*myo*-1,2,6-inositol triphosphate significantly reduced the NPY-induced feeding response during the initial 5 h period following injection.

In 1990 Doughty reported the irreversible α_1-adrenergic antagonist benextramine (**2a**) to be a long-lasting antagonist of NPY in vascular and neuronal tissue [141]. When administered at 15 mg/kg, i.v Benextramine inhibited the NPY-induced pressor response in pithed Sprague-Dawley rats. with a calculated ED_{50} of 0.46 nmol/kg at the 90 min. time point. In NPY binding experiments, benextramine was found to irreversibly inhibit [^{3}H]NPY binding in rat brain membranes suggesting the compound could covalently link to a site on the receptor. Considering the disulfide bond present in benextramine, this result was not unanticipated. Subsequent selectivity studies suggested that benextramine was a mixed Y_1/Y_2 antagonist in various peripheral [142, 143] and neuronal tissues [144, 145].

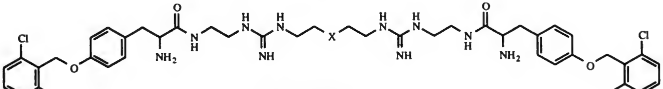
Structure-activity studies around the terminal aryl portion of benextramine (**2a**) were conducted by Doughty [146]. As shown in Table 2, compounds with a π-rich aromatic rings substituted in the *meta*-position were optimal for good NPY binding affinity. The 3-methoxyphenyl derivative **2d** was approximately three times more potent than the parent structure **2a** having the 2-methoxyphenyl terminus. Both phenolic and methyl ether substituted aromatics were equally active. Binding affinity diminished on changing to a 3-chloro substituent. The



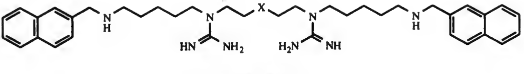
D-*myo*-1,2,6-inostol triphosphate (**1**)



benextramine (2a)



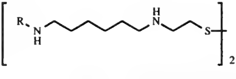
SC3117, X=S (**3a**)
SC3199, X=CH_2 (**3b**)



CC217, X=S (**4a**)
CC2137, X=CH_2 (**4b**)

Chart 1

Table 2. Benzylic Benextramine Derivatives 2a-2l



Cmpd	R	IC$_{50}$ (μM)^a
2a	2-MeOPhCH$_2$	57.6
2b	2-HOPhCH$_2$	50.6
2c	2-ClPhCH$_2$	79.9
2d	3-MeOPhCH$_2$	21.5
2e	3-HOPhCH$_2$	20.3
2f	3-ClPhCH$_2$	46.7
2g	4-MeOPhCH$_2$	44.1
2h	4-HOPhCH$_2$	161.5
2i	4-ClPhCH$_2$	37.8
2j	2-naphthyl-CH$_2$	31.3
2k	PhCH$_2$	191
2l	H	>1400

[a] The value represents the concentration of 2 needed to produce a half-maximal displacement of [^{3}H]NPY specific binding from benextramine sensitive NPY binding sites in rat brain membranes.

2-naphthyl derivative **2j** (IC$_{50}$ 31.3 μM) also demonstrated equipotent affinity at the receptor suggesting bulk tolerance for large lipophilic substituents. Apparently the aromatic ring portion of the molecule is critical for recognition since its removal afforded a compound devoid of NPY binding (2l, IC$_{50}$ >1400 μM). This is informative since it also suggests the reactive disulfide moiety in the benextramine series is not in itself the feature responsible for the observed NPY binding. There was also a clear divergence of NPY and α_1-adrenergic SAR indicating that these two pharmacological effects are indeed separable.

More recently, Doughty prepared several benextramine analogs to closely mimic some of the essential polar features of the NPY molecule. In order to mimic the N- and/or C-terminal Tyr^1 and Tyr^{36} groups of NPY, SC3117 (**3a**) and SC3199 (**3b**) were designed to provide a terminal tyrosine moiety and an internal guanidine group to simulate the Arg^{33} and Arg^{35}

Table 3. Guanidine Incorporating Benextramine Derivatives 3 and 4

Cmpd	NPY Binding (IC$_{50}$, μM)^a	*In Vitro* Antagonism (IC$_{50}$, μM)^b
2a	56.0	2.9
3a	18.5	0.0431
3b	45.4	0.414
4a	14.8	*c*
4b	18.4	*d*

[a] The value represents the concentration of 2 needed to produce a half-maximal displacement of [^{3}H]NPY specific binding from benextramine sensitive NPY binding sites in rat brain membranes. [b] Inhibition of rat femoral artery constriction induced by near maximal concentrations of NPY. [c] More potent than **2a**, **3a**, and **3b** in this assay. [d] 1000-fold more potent than **2a** in decreasing NPY-induced contractions in this tissue.

guanidinium positions in NPY [147]. Similarly, the design features of CC217 (**4a**) and CC2137 (**4b**) include external guanidine groups with a napthyl aromatic terminus [148]. Both disulfide and the corresponding carbon-linked congeners were prepared to examine the effect of the reactive disulfide bond on covalent binding. As shown in Table 3, all of the analogs showed enhanced potency in the NPY binding experiments in rat brain. Their relative binding potencies varied from 1.2 to 3.8-fold vs. benextramine binding with compounds **3a**, **4a**, and **4b** showing the greatest effect. These latter analogs were found to be selective for benextramine sensitive sites in rat brain.

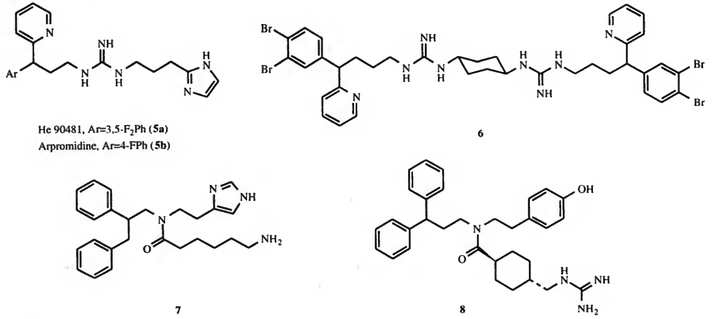
All of the guanidine containing benextramine analogs inhibited NPY-induced contractions in vascular smooth muscle (rat femoral artery) with IC_{50}'s of <1 μM. Surprisingly, relative potencies for functional inhibition of NPY-induced contractions in vascular tissue were much greater than would have been predicted from their relative binding affinities in rat brain membranes. Thus, SC3117 demonstrated a 67-fold potency enhancement in vascular tissue vs. benextramine while being only 3-fold more potent in the binding assay. Similarly, CC2137 was found to be greater than 1000-fold more potent than benextramine in this assay (data not shown). Since none of the guanidine containing analogs demonstrated any α-adrenergic inhibition in vascular tissue, Doughty speculated the disparity in NPY effects could be due to structural differences between brain and vascular NPY receptors. Functional experiments with CC2137 in the rat femoral artery found the compound had no effect on the inhibition of contractions induced by the Y_1 selective agonist [Leu^{31},Pro^{34}]NPY. However CC2137 did inhibit the contractions induced by the selective Y_2 agonist $NPY_{13\text{-}16}$ (pA_2 9.2) suggesting it is selective for the Y_2 receptor in this tissue. A similar result has also been observed with SC3117 [149].

The heterocyclic guanidine derivative He 90481 (**5a**) related to the histamine H_2 antagonist arpromidine (**5b**) has been reported to be a weak but competitive antagonist of NPY-induced Ca^{2+} efflux into HEL cells (pA_2 4.43) [150]. Unfortunately the compound is not selective for the NPY receptor since it also has potent modulatory effects at both histamine H_1 and H_2 sites [151]. Structure-activity studies found that the basicity of the central guanidine is critical for NPY activity and that lipophilic substituents attached to the guanidine moiety enhance NPY potency [152].

Knieps has described a series of bisguanidine derivatives related to He 90481. The bisguanidines were prepared as a result of modeling studies which suggested the incorporation of an additional guanidine group to mimic the Arg^{33}, Arg^{35} portions of NPY would be beneficial for activity [153]. From this series, emerged a number of compounds (e.g. **6**) which showed enhanced Y_1 potency in the HEL cell assay as compared to He 90481. The series also demonstrated diminished histamine activity as compared to He 90481. From the results of the study using rigid linkers, they suggested a 8 Å distance between the basic guanidine moieties was optimal for NPY activity. This is in agreement with earlier modeling studies which found a 7-8Å distance between the guanidinium positions of Arg^{33} and Arg^{35} in the putative binding confirmation of NPY [154].

More recently, Mueller has described a series of analogs in which the central guanidine portion of He 90481 has been replaced with an amide linkage and moved the required basic site to a terminal position to more closely mimic the C-terminal arginine units of NPY [155]. The most active analog in the series is the imidazole **7** which was found to have an IC_{50} of 0.9 μM against NPY-induced Ca^{2+} efflux into HEL cells. Their modeling studies suggested that the two basic positions of **7** (the terminal primary amine and the imidazole) to interact with the Arg^{33} and Arg^{35} of NPY, respectively. Surprisingly, the *in vitro* activity was considerably diminished when **7** was made more arginine-like by conversion of the terminal primary amine group to a guanidine. The authors proposed that additional branching of the guanidine group might be responsible for the reduction in activity. The authors suggest that reduced basicity of these primary amine compounds might enhance their ability to be developed as centrally active NPY antagonists.

Mueller also reported in the same paper that replacement of the imidazole portion of the molecule with a phenolic terminus was well tolerated despite eliminating one of the required basic



He 90481, Ar=3,5-F_2Ph (**5a**)
Arpromidine, Ar=4-FPh (**5b**)

6

7

8

Chart 2

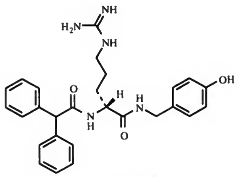
Table 4. **Y_1 and Y_2 Binding Profile of BIBP 3226 (9).**

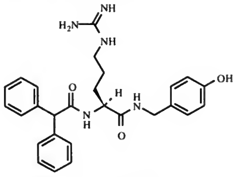
Receptor Type (cell or tissue)	BIBP 3226 (K_i, nM)
human Y_1 (SK-N-MC)	5.1
human Y_1 (CHO)	0.47
rat Y_1 (HEK 293)'	13.9
rat Y_1 (cortex)	6.8
human Y_2 (SMS-KAN)	>10,000
rat Y_2 (hippocampus)	>10,000
rabbit Y_2 (kidney)	>10,000
dog Y_2 (adipocytes)	>10,000

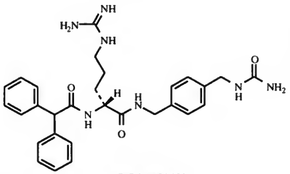
sites in the molecule. One of their more potent analogs in the series was the phenolic guanidine **8** which was found to have an IC_{50} of 11 μM in their assay. They suggest that, in this latter series, the basic guanidine and acidic phenol components possibly mimic C-terminal Arg^{35} and Tyr^{36} positions of NPY. No anorectic activity was reported on any of the analogs.

BIBP 3226 (**9**), the first highly potent sub-micromolar Y_1 antagonist, was reported by Karl Thomae scientists in 1994 [156]. The compound was identified as a result of a rationally designed program to modify small C-terminal portions of NPY. Two recent reviews have appeared which summarize both the pharmacology and structure-activity studies of BIBP 3226 [157,158]. Binding experiments with BIBP 3226 showed that it was a selective Y_1 antagonist [159]. The compound was found to bind potently to both the human and rat Y_1 receptors and have no affinity for the human, rat, or dog Y_2 receptors (Table 4). BIBP 3226 was shown to be a full antagonist in both neuronal cells and peripheral tissues. The compound inhibited the NPY-induced increase of intracellular Ca^{2+} in SK-N-MC cells (K_b 48.9 nM), and the NPY-induced increase in perfusion pressure in isolated perfused rat kidneys (IC_{50} 26 nM). The compound had no Y_2 receptor antagonism since it did not block NPY-induced contractions in isolated and electrically stimulated rat vas deferens. BIBP 3226 was confirmed to be an antagonist *in vivo* since it inhibited the NPY-induced rise in blood pressure in pithed rats (ID_{50} 0.11 mg/kg, i.v.).

Structure-activity studies around the BIBP 3226 chemotype revealed that the pendant phenol and its correct spatial


BIBP 3226 (**9**)


BIBP 3435 (**10**)


BIBO 3304 (**11**)

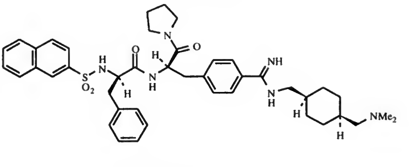

SR 120819A (**12**)

Chart 3

positioning are important for potent Y_1 affinity. The central arginine portion of BIBP 3226 is believed to mimic Arg^{35} in NPY since its removal or modification of its position relative to the backbone diminished binding affinity. Both aromatic rings at the geminally substituted, diphenylacetic acid portion of BIBP 3226 are also important for binding since their removal results in considerable loss of potency in the binding assay. Interestingly, the chirality at the unnatural R-arginine portion of BIBP 3226 appears to be a critical feature for receptor recognition since the enantiomeric analog BIBP 3435 (**10**) derived from naturally occurring S-arginine is devoid of Y_1 binding affinity in SK-N-MC cells [159].

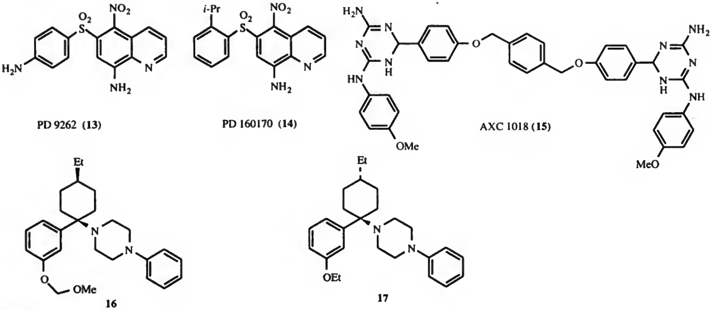
The anorectic activity of BIBP 3226 remains controversial. Scientists at Karl Thomae were not able to unambiguously demonstrate a feeding effect in rats because of serious barrel-rolling side effects that were observed upon i.c.v. administration of the compound [159]. However, O'Shea et al. have reported that BIBP 3226 significantly attenuates NPY-induced feeding (1.2 nmol) in rats when a dose of 60 nmol, i.c.v. was administered [160]. We and others have been unable to demonstrate anorectic effects in similar rat models following i.c.v. or PVN administration [A. Ortiz, personal communication]. It should be noted that i.c.v. administration of BIBP 3226 in rats does result in an anxiogenic-like effect which can be blocked by diazepam [161]. This suggests that BIBP 3226 demonstrates Y_1 receptor-mediated behavioral effects in an *in vivo* situation. Lastly, Karl Thomae has recently reported that i.c.v. administration of the close structural analog BIBO 3304 (**11**) significantly inhibited the NPY-, NPY(3-36)-, and $[Leu^{31},Pro^{34}]$NPY-induced feeding response in satiated rats after PVN administration [162,163]. In binding experiments, the compound was significantly more potent than BIBP 3226 in a SK-N-MC cells assay using $[^{125}I]$Bolton-Hunter NPY (IC_{50} 0. 37 nM). They also showed that BIBO 3304 could significantly reduce hyperphagia in rats which had been fasted for 24 h, and delay body weight gain when administered chronically for 1 week into the third ventricle of Zucker and Wistar rats.

SR 120819A (**12**) was disclosed by Sanofi scientists in 1995 and reported to be the first orally active NPY antagonist for the possible treatment of hypertension [164]. The pharmacology of SR 120819A has been recently reviewed [165]. Binding experiments in SK-N-MC cells found SR 120819A to be competitive with $[^{125}I]$PYY and to display potent affinity at the Y_1 receptor (K_i 15 nM). The compound showed no affinity for the Y_2 receptor in rabbit kidney or human cortex preparation, or functional modulation of the Y_3 receptor by contractile response in a rat colon assay. SR 120819A also does not interact with the Y_4 or Y_5 receptor subtypes, nor with a variety of other nonrelated receptors [166]. The compound was found to be a full antagonist in a forskolin-stimulated adenylate cyclase assay in SK-N-MC cells.

The oral activity of SR 120819A was discerned in an antihypertensive assay. SR 120819A blocked the $[Leu^{31},Pro^{34}]$NPY-induced pressor response in anesthetized guinea pig dose dependently when administered either by an i.v. route (0.1-1.0 mg/kg) or an oral route (5 and 10 mg/kg). The antihypertensive effects were generally long-lasting (>3 h). The CNS activity of 120819A is unknown since its ability to penetrate the blood-brain-barrier is low. When dosed i.p. at 5 mg/kg, the compound showed no significant antagonism of NPY or $[Leu^{31},Pro^{34}]$NPY-induced behavioral effects.

High throughput screening efforts at Parke-Davis recently identified the arylsulfonyl quinoline derivative PD 9262 (**13**, K_i 282 nM, ^{125}I-PYY in SK-N-MC cell membranes) [167]. Subsequent structure-activity studies showed that substitution at the 2-position on the phenylsulfone portion of the molecule enhanced Y_1 binding. The studies ultimately resulted in the identification of PD 160170 (**14**) which displayed a K_i of 48 nM in their Y_1 receptor binding assay. The compound was shown to be selective for the Y_1 over the Y_2 receptor and found to be a full antagonist in a functional forskolin-stimulated cAMP assay in SK-N-MC cells (IC_{50}, 0.3 nM). There have been no reports on the effect of PD 160170 in feeding studies.

A series of non-peptide Y_1 antagonists have been reported by scientists at Alanex [168]. Their compounds were designed based on a hypothetical pharmacophore model which was derived by computer analysis of known peptide and peptide-like NPY antagonists. One of the more potent compounds in their series was the bis-dihydrotriazine derivative AXC 1018 (**15**)



PD 9262 (**13**) PD 160170 (**14**) AXC 1018 (**15**)

16 **17**

Chart 4

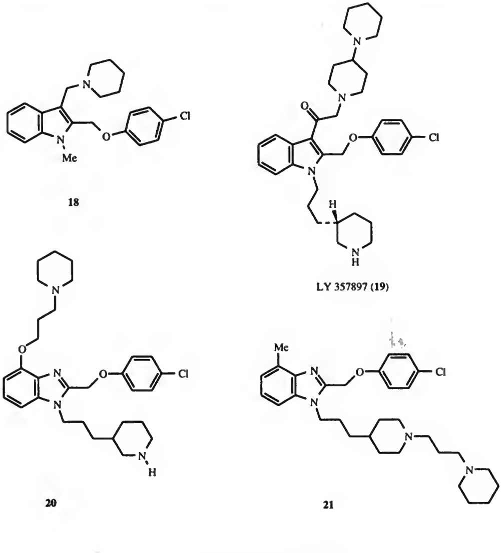
which displayed an IC_{50} of 150 nM in a SK-N-MC cell based assay. The compound was found to be a full antagonist in their functional norepinephrine-stimulated cAMP assay in SK-N-MC cells (K_b 400 nM). Unfortunately, the bis-dihydrotriazine class of NPY compounds were not developed further since they were found to produce undesirable side effects in animal studies. However, Alanex has more recently pursued a combinatorial strategy to identify different NPY chemotypes and have reported a selective Y_1 ligand with 30 nM affinity (AXC 4141, structure not disclosed).

Scientists at Neurogen Corporation have disclosed a series of benzylamine derivatives which display high Y_1 binding affinity [169]. Their most potent compound was the *cis*-cyclohexane derivative **16** which displayed an IC_{50} of 39 nM in a Y_1 binding assay in SK-N-MC cells. The stereochemical relationship between the 4-alkyl substituent and the aryl piperidine on the cyclohexane appears to be important since a corresponding *trans* derivative **17** displayed diminished potency in the binding assay (IC_{50} 525 nM). No feeding data on any of the compounds was disclosed.

The first subnanomolar NPY antagonist was reported by scientists at Eli Lilly [170]. Biased screening efforts identified the low molecular weight trisubstituted indole derivative **18** as a Y_1 hit (K_i 2.1 µM) in their binding assay using cloned human Y_1 receptors expressed in AV-12 cells. Subsequent structure-activity studies at both the N-1 and C-3 positions of the indole resulted in the identification LY 357897 (**19**) which was found to have a K_i of 0.75 nM in their Y_1 assay. To date, LY 357897 represents the most potent, non-peptidic Y_1 receptor antagonist known. Scientists at Eli Lilly ascribe the 2800-fold potency improvement relative to **18** as a probable result of the additional basic sites on both N-1 and C-3 side chain sites and an optimal spatial arrangement from the C-3 carbonyl. The chirality at the C-3 position of the piperidine does not appear to be critical since the enantiomeric R- analog was still found to be quite potent in their binding assay (K_i 1.4 nM).

LY 357897 showed no appreciable binding affinity for the Y_2, Y_4, or Y_5 receptor subtypes (K_i's >10,000 nM) , and was found to be a full antagonist in an NPY-induced forskolin-stimulated cAMP assay (K_i 1.8 nM). The compound was also found to inhibit NPY-induced intracellular Ca^{2+} mobilization in SK-N-MC cells (K_i 3.2 nM). Lastly, LY 357897 was found to block the NPY-induced feeding response in mice when administered into the lateral ventricle in a dose-dependent manner (ED_{50} 17 nmol). No neuromuscular dysfunctions were observed suggesting the inhibition of NPY-induced feeding effect was mediated by Y_1 antagonism. Unfortunately LY 357897 was not orally or subcutaneously bioavailable which precluded its study by these routes of administration.

A more recent paper from Eli Lilly has disclosed a series of similar benzimidazole Y_1 antagonists [171]. The compounds **20** and **21** displayed K_i's of 2.0 and 4.5 nM, respectively, in their



18

LY 357897 (19)

20

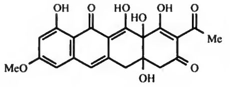
21

Chart 5

AV-12 cell binding assay. Benzimidazole **21** was subsequently found to reverse NPY-induced inhibition of forskolin-stimulated cAMP (K_i 46 nM) and to inhibit NPY-induced intracellular Ca^{2+} accumulation in SK-N-MC cells (K_i 26 nM). No feeding data on any of the compounds was disclosed.

Y_2 Receptor Antagonists

Although a number of peptidic Y_2 receptor agonists have been described in the literature, there is very little information on non-peptidic Y_2 receptor antagonists. Scientists at Bristol-Myers Squibb have isolated BMS-192548 (**22**) from the extract of *Aspergillus niger* WB2346 [172,173]. BMS-192548 was found to inhibit [^{125}I]PYY binding in SMS-KAN cells (IC_{50} 27 µM). The compound was not selective for the Y_2 receptor subtype since it also inhibited [^{125}I]PYY binding in SK-N-MC cells (IC_{50} 24 µM) with equal potency. The compound was not examined in any functional assays to determine its agonist or antagonist properties.



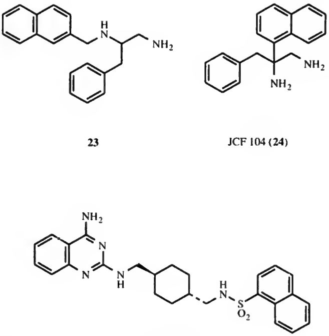
BMS-192548 (**22**)

A number of the benextramine analogs which were discussed in the previous section are also mixed Y_1/Y_2 antagonists. Doughty reported that CC2137 (**4b**) was found to potently antagonize NPY(13-36)-induced contractions in the rat femoral artery (IC_{50} 2.0 nM) [174].

Y_5 Receptor Antagonists

Since the discovery of the Y_5 receptor in 1996, several antagonists have been reported. Synaptic Pharmaceutical disclosed the diamine **23** in their original patent application [175]. The diamine displayed a K_i of 19 µM in a binding assay using the human Y_5 receptor stably expressed in a mammalian cell line. No anorectic activity of the compound was reported. A similar diamine derivative, JCF 104 (**24**) was reported to functionally inhibit human PP-induced contractions in rabbit ileum (IC_{50} 1.7 µM) and explained in terms of a Y_5 mechanism [176].

The most potent NPY Y_5 antagonist identified to date is CGP 71683A (**25**) [177]. Novartis scientists have reported that CGP 71683A displays high affinity for the cloned human and rat Y_5 receptors (IC_{50}'s of 2.0 and 1.4 nM, respectively) [178]. The compound was found to antagonize NPY-induced Ca^{2+} transients in LM(tk-) cells transfected with the human Y_5 receptor (IC_{50} 6 nM). The compound has also been examined in a number of feeding models. At 10 mg/kg, i.p. CGP 71683A significantly inhibited both NPY-induced food intake in satiated rats 2 h after dosing and food intake in fasted rats for 24 h after dosing. It also significantly reduced food intake in *ob/ob* mice and Zucker obese rat models.



23

JCF 104 (**24**)

CGP 71683A (**25**)

Chart 6

A potent and orally bioavailable Y_5 antagonist has also been reported by Merck [179]. L-152,804 (structure not disclosed) displayed high affinity at both the human Y_5 and rat Y_5 receptors (K_i's of 27 and 31 nM, respectively) and blocked the NPY-induced increase of intracellular Ca^{2+} levels in Y_5 expressing cells. L-152,804 showed no affinity for the human Y_1, Y_2, and Y_4 receptor subtypes. The compound significantly blocked bovine PP induced food intake in satiated Sprague-Dawley rats when administered by i.c.v. or oral routes. Interestingly, L-152,804 did not inhibit NPY-induced feeding in the same model, nor did the compound inhibit spontaneous food intake in either Zucker fatty rats or *db/db* mice when given orally at 100 mg/kg.

Which NPY Receptor Exerts the Effect of NPY on Feeding?

The previous sections provide evidence that increased NPY receptor hypothalamic levels of NPY contribute to the orexigenic symptoms seen in animal models of obesity and that various antagonists can inhibit its actions. Until the early 1990's it was believed that the orexigenic effect of NPY was mediated by the Y_1 receptor [180]. The Y_2 receptor has been excluded based on its distribution and its pharmacology, namely that it readily recognizes C-terminal NPY fragments as NPY(13-36), which have little effect on food intake [181]. The Y_3 receptor can be also easily discounted since it does not recognize PYY while PYY is a relatively potent stimulator of food intake [182]. Similarly the involvement of the Y_4 receptor is unlikely, since the most potent agonist of this receptor, PP, has much less effect on food intake than NPY [113]. The involvement of the y_6 receptor is questionable, since this receptor appears to be non-functional in humans [118,119]. At present, the only two receptors which are the most likely candidates to conduct NPY's effect on feeding are the Y_1 and Y_5 receptors, whose pharmacology is also quite similar (Table 1).

As mentioned previously, the selective Y_1 antagonists 1229U91, BIBO 3304, and LY 357897 potently inhibit NPY-induced feeding in rodents after central administration which argues for the feeding response to be mediated at least in part through the Y_1 receptor. However, Stanley et al. were the first to suggest that the effect of NPY on feeding is mediated through an "atypical" Y_1 receptor [182]. The argument came from the observation that NPY(2-36) was as potent in enhancing food intake as NPY itself, but had much lower affinity for the Y_1 receptor than NPY itself [183,184]. Additionally, a cyclic analog of NPY was shown to have high affinity for the Y_1 receptor as an agonist *in vitro* while it failed to stimulate food intake *in vivo* [185]. O'Shea et al. [160] showed that [Pro^{34}]NPY gave the same maximal inhibition of adenylate cyclase in SK-N-MC cells which known to express the Y_1 receptor, while it only stimulated feeding approximately half as much as NPY itself. Another important observation by the same authors was the effect of [Pro^{34}]NPY(3-36). The peptide was unable to inhibit adenylate cyclase at the same concentration at which [Pro^{34}]NPY completely inhibited it, although the two compounds were equipotent in stimulating food intake. It is of note that Y_1 expression in the hypothalamus, which is the site of NPY's action, is relatively low in both rat and human brain [186,187].

Studies utilizing antisense oligodeoxynucleotides to reduce the expression of the Y_1 receptors in the brain have provided contradicting evidence on the role of the Y_1 receptor in feeding. One study suggests that central administration of Y_1 receptor antisense oligodeoxynucleotide into rat hypothalamus reduces food intake [188], while another found that it resulted in an anxiogenic effect [189] and paradoxically an enhancement of food intake [190]. Collectively these findings suggest that receptors other than the Y_1 are possibly involved. Never-the-less, the data with selective Y_1 receptor antagonists provides compelling evidence that the Y_1 receptor is involved in the regulation of food intake. However, it should be emphasized that the data do not necessarily preclude the involvment of other NPY receptors.

The pharmacology of the recently discovered Y_5 receptor suggests that it may responsible for NPY's feeding effect [113]. The authors report positive correlation between the stimulation of food intake and inhibition of forskolin-stimulated cAMP response in Y_5 receptor transfected cells by different NPY analogs. The feeding response was also stimulated, although only at high concentration (2 nmols vs 0.3 nmols), by the Y_5 selective agonist [$D\text{-}Trp^{32}$]NPY. Even though NPY(13-36) has relatively high affinity for the Y_5 receptor (20 nM), it failed to stimulate food intake [180] which creates some doubts on the suggestion that the Y_5 receptor is the "feeding receptor". Never-the-less, as described previously, the selective Y_5 antagonist CGP 71683A demonstrated efficacy in a number of rodent feeding models suggesting there is an involvement of the Y_5 receptor with feeding. Moreover, repeated i.c.v. injections of Y_5 antisense oligodeoxynucleotides significantly reduced basal, NPY-induced, and fasting-induced food intake [191]. Taken together a role for the Y5 receptor in regulating food intake is inferred.

The scientific community hoped that the recent production of NPY, Y_1 and Y_5 receptor knock-out mice would have shed more light on the elucidaing the role of NPY and its receptors on food intake and energy expenditure. Most surprisingly NPY knock-out mice lacked the expected phenotype. Their food intake and body weight were not significantly different from that of the wild-type animals [192]. Additionally their endocrine systems responded normally to starvation demonstrating that food intake and energy expenditure are redundantly regulated to a high degree in the body. NPY deficient mice however had an enhanced response to leptin. NPY deficiency partially reduced the obesity syndrome caused by leptin deficiency in *ob/ob* mice, as the double knock-out mice ate less, were less prone to diabetes and were more fertile than their *ob/ob* littermates [192].

Y_5 knock-out mice were remarkably normal in terms of their food intake, body weight, and fertility. However, NPY and other PP-induced feeding was less pronounced in Y_5 receptor deficient mice than in wild types, suggesting that in addition to the Y_5 receptor other NPY receptors might have a role in the regulation of food intake [193]. Similarly, Y_1 receptor deficient mice also appeared normal and fertile. Preliminary studies suggested that the homozygous animals have a defect in general metabolism which results in excess fat accumulation [194]. Blood pressure, heart rate and cardiovascular response of the Y_1 receptor deficient mice to norepinephrine was normal. NPY-induced feeding appeared to be reduced in these animals compared to the wild types, and the knock-out animals cumulatively move less during the dark cycle than their wild type counterparts (T. Pedrazzini, presentation on the Second International NPY Conference, London, UK, 1997). These findings again suggest that multiple receptors may be involved in the regulation of NPY-induced food intake.

Genetic studies conducted in a morbidly obese population of French Caucasians investigated the potential implication of NPY, and the Y_1 and Y_5 receptors in the development of morbid human obesity [195]. The study found no evidence of linkage between morbid obesity and NPY or Y_1/Y_5 receptor regions in the investigated population, and similarly no mutations in the coding regions of the NPY and the Y_1 receptor gene among the tested obese patients. These results should be interpreted with caution, however, since obesity is an extremely complex disease with unknown etiology. The fact that one studied population did not show mutations or linkage in the investigated loci does not mean that these genes and their products are not involved in the regulation of energy intake and expenditure or in the development of general obesity. The authors suggested that their data was consistent with the findings of Sipols et al. [196] in baboons which showed that centrally administered NPY had no effect on food intake. It should be noted that the paradigm used in the baboon study was not optimal for studying the efficacy of NPY in these animals. The baboons received i.c.v. NPY after a 16.5 hr fast at which time their NPY levels were already elevated and therefore exogenous NPY could not possibly enhance their food intake compared to the control group. The data provided by this study is at best inconclusive. As previously noted primate study in rhesus monkeys found that NPY is a potent orexigenic agent in that species, too [48].

Numerous studies provide evidence that NPY exerts its action on the regulation of energy balance via Y_1, Y_5, or yet another unknown Y_1-like receptor. It is also possible based on the response of Y_1 and Y_5 receptor deficient mice to centrally administered NPY that there are perhaps more than one NPY receptors which co-participate in the regulation of energy balance. Further studies with highly selective ligands will undoubtedly furnish the answers to these questions. However, the acid test of any NPY-ergic antagonist(s) for the possible treatment of human obesity awaits demonstration of efficacy in a clinical setting.

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List of Abbreviations

CRF = Corticotropin Releasing Factor
i.c.v. = Intracerebroventricular
NIDDM = Non-insulin Dependent Diabetes Mellitus
NPY = Neuropeptide Y
PCR = Polymerase Chain Reaction
PP = Pancreatic Polypeptide
PVN = Paraventricular Nucleous
PYY = Pancreatic Polypeptide Y
VMN = Ventromedial Nuclei

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